Universidade de Aveiro Departamento de Biologia
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Pedro Araújo Carvalho

Impacto da idade em proteínas de sinalização de espermatozoides

Impact of age on spermatozoa signaling proteins

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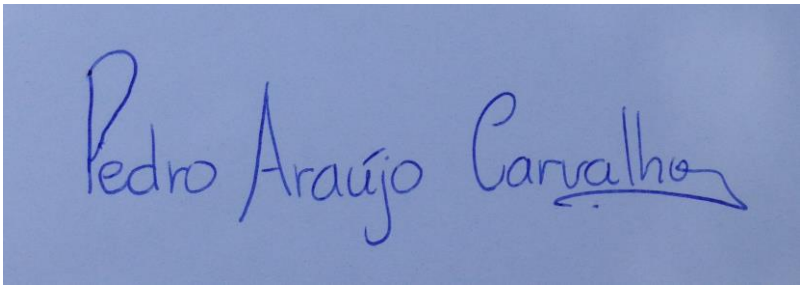

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Margarida Sâncio da Cruz Fardilha, Professora Auxiliar do Departamento das Ciências Médicas da Universidade de Aveiro e co-orientação da Doutora Joana Vieira Silva, Investigadora do Instituto de Investigação e Inovação em Saúde.

Dedico esta dissertação aos meus pais, Augusto e Virgínia.

o júri

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Agradecimentos

À Professora Doutora Margarida Fardilha, por todo os ensinamentos que me transmitiu ao longo do meu percurso académico. Destaco e agradeço a sua franqueza, humildade e disponibilidade.

À Doutora Joana Vieira Silva, pela enorme ajuda e total disponibilidade que demonstrou bem como todos os ensinamentos que me passou de forma coerente. Foi uma das pessoas mais altruístas e humildes que conheci neste último ano académico e sem ela, esta dissertação não era possível.

À Dra. Madalena Cabral, pois sem ela não teria tido a oportunidade de estagiar na COGE e de proceder à recolha das amostras para a realização deste trabalho. Aprendi com ela que é possível ser-se profissional, ambicioso e curioso. É um exemplo para mim pois demonstrou-me a paixão que tem por este tema que tanto me fascina.

À Dra. Carla Leal, pelo companheirismo e boa-disposição. Por ser uma das pessoas mais divertidas que conheci em ambiente laboratorial. Agradeço também os ensinamentos que me transmitiu.

Ao Miguel, pela ajuda na análise estatística.

A todos os meus amigos, em particular à Luísa e à Diana, por estarem sempre presentes nos momentos mais difíceis e nelas encontrar sempre uma palavra amiga de força e encorajamento.

A toda as minhas colegas de trabalho e alunos do Cubo Mágico pela paciência e persistência.

Por fim e em especial, aos meus pais, Augusto e Virgínia, e aos meus irmãos, André e Andreia, pela motivação e por sempre acreditarem em mim. Agradeço as palavras de consolação e por sempre me motivarem a prosseguir mais além e por acreditarem na minha paixão.

palavras-chave

(in)fertilidade masculina, espermatozoide, transdução de sinal, PRAS40, P70 S6 quinase.

resumo

A infertilidade pode ser definida como a incapacidade de conceber uma gravidez após pelo menos doze meses de relações sexuais regulares desprotegidas. O fator masculino encontra-se envolvido em aproximadamente 50% dos casos de infertilidade conjugal, sendo exclusivamente responsável em aproximadamente 20% dos casos. O adiamento da paternidade sugere que a idade pode ser uma causa de problemas reprodutivos. Embora esteja bem documentado que as mulheres apresentam um declínio na fecundidade com o avançar da idade, os dados disponíveis sobre os efeitos do envelhecimento na fertilidade masculina mostram uma maior disparidade. Apesar de ser cientificamente consensual que a idade masculina é um fator importante, muito pouco se sabe sobre os mecanismos moleculares subjacentes à relação entre idade masculina e fertilidade reduzida.

O objetivo deste estudo foi avaliar o impacto do envelhecimento em espermatozoides humanos. Para esse fim, os parâmetros seminais básicos e os níveis de 18 proteínas de sinalização foram analisados em 31 homens que recorreram a Técnicas de Procriação Medicamente Assistida (PMA).

O presente estudo revelou que a idade do sexo masculino estava associada à percentagem de defeitos da peça intermediária e à presença de citoplasma residual em excesso (CRE) em espermatozoides.

Este estudo revelou ainda que o nível de duas fosfoproteínas de espermatozoides humanos, PRAS40 (Thr246) e P70 S6 quinase (Thr389) apresentaram uma correlação significativamente negativa ($p<0,01$) com idade masculina. Assim, pôde-se concluir que essas duas fosfoproteínas podem ser consideradas bons marcadores para a monotorização do declínio da fertilidade masculina intrínseca ao processo de envelhecimento. Contudo, mais estudos com um maior número de amostras deverão ser realizados.

keywords male (in)fertility, spermatozoa, signal transduction, PRAS40, P70 S6 kinase.

Abstract

Infertility is defined as the inability to achieve a pregnancy after twelve or more months of unprotected regular intercourse. The male factor is involved in approximately 50% of the cases of conjugal infertility, being exclusively responsible in approximately 20% of the cases. The postponement of paternity suggests that age may be a cause of reproductive problems. While it is well documented that women have a decline in fecundity with age, the data available regarding the effects of age on male fertility show a wider disparity. Despite the scientific consensus that male age is an important factor, very little is known about the molecular mechanisms underlying the connections between male age and reduced fertility.

The aim of this study was to evaluate the impact of aging on human spermatozoa. To that end, the basic seminal parameters and the levels of 18 signaling proteins were analyzed in 31 men who resort to Assisted Reproductive Techniques (ART).

The present study revealed that male age was associated with the percentage of midpiece defects and the presence of excess residual cytoplasm (ERC) in spermatozoa.

This study also showed that the level of two phosphoproteins in human spermatozoa, PRAS40 (Thr246) and P70 S6 kinase (Thr389), presented a significant negative correlation ($p<0.01$) with male age. Therefore, these two phosphoproteins may be good markers to monitor the male fertility decline intrinsic to the aging process. Although more and larger studies must be conducted.

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List of abbreviations

AC	Adenylyl cyclase
AI	Artificial Insemination
AKAPs	A-kinase anchoring proteins
AMP	Adenosine monophosphate
ART	Assisted Reproduction Technology
ATP	Adenosine-5-triphosphate
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CatSper channel	Cation channels of sperm
CD45	Leukocyte common antigen
CDK16	Cyclin-dependent kinase 16
COGE	Clínica Obstétrica e Ginecológica de Espinho
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
FS	Fibrous sheet
FSH	Follicle-stimulating hormone
G6PDH	Glucose-6-phosphate dehydrogenase
GH	Growth hormone
Glu	Glutamic acid
GPR18	G protein-coupled receptor 18
GPX5	Type 5 glutathione peroxidase
GSK3	Glycogen synthase kinase 3
H_2O_2	Hydrogen peroxide
hCG	Human chorionic gonadotropin

HCO_3^-	Bicarbonate
iBiMED	Institute for Research in Biomedicine
ICSI	Intracytoplasmic sperm injection
IVF	*In vitro* fertilization
IZUMO1	Sperm-egg fusion protein 1
LH	Luteinizing hormone
MAPK/ERK	Mitogen-activated protein kinase
MS	Mitochondrial sheath
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NO^-	Nitric oxide
O^{2-}	Superoxide
ODFs	Outers dense fibers
P70 S6 Kinase	Ribosomal protein S6 kinase beta-1
PDE	Phosphodiesterase
pH	Potencial of Hydrogen
PI3	Phosphoinositol 3-phosphate
PIP2	Phosphotidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKB/AKT	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PPP1	Phosphoprotein phosphatase 1
PRAS40	Proline-rich AKT1 substrate 1
Raf	Rapidly accelerated fibrosarcoma
ROS	Reactive oxygen species
RPS6	Ribossomal protein S6
sAC	soluble adenylyl cyclase
Ser	Serine
sFUT5	Sperm fucosyltransferase-5

Shc	Src homology collagen
SOC	Store-operated channels
Thr	Threonine
tRNA	Transfer ribonucleic acid
Tyr	Tyrosine
WHO	World Health Organization
WR	Working Reagent
ZP3	Zona pellucida sperm-binding protein 3

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1. General Introduction and Objective

The male reproductive system consists of two testicles (male gonads), a system of genital ducts, the accessory glands (prostate, bulbourethral gland and seminal vesicles) and the penis. The testicles produce male gametes (spermatozoa) through spermatogenesis and secrete male sex hormones, testosterone.

1.1. Testis

The testicles are the male gonads, responsible for the synthesis of male germ cells as well as sex hormones. They are oval structures involved by the scrotum and suspended by the spermatic cord (Figure 1). The testicles are surrounded by the tunica albuginea, that contains abundant contractile elements (Middendorff et al., 2002). The testicular parenchyma is composed of seminiferous tubules constituting the testicular pulp, where the production of spermatozoa occurs, and is supported by loose connective tissue (Kerr, 1992).

1.1.1. Leydig cells

Cells with endocrine function are found adjacent to the blood vessels, the Leydig cells. They are responsible for the production of the male sex hormone, testosterone. There are two generations of Leydig cells – fetal and adult (Prince, 2001). The fetal generation of Leydig cells (from birth to the first year of age) resulting from Chorionic Gonadotropin (hCG) stimulation have round nucleus, abundant smooth endoplasmic reticulum and mitochondria with tubular cristae (Nistal et al., 1986). The adult generation, under the stimulation of the Luteinizing Hormone (LH) has the role of producing testosterone, from puberty and during the entire life (Benton, Shan and Hardy, 1995). Human Leydig cells contain large Reinke crystalloids of variable size and number (Kerr, 1991). The high levels of circulating testosterone at puberty cause the testis cords to canalize and become the seminiferous tubules (Clermont and Huckins, 1961).

1.1.2. Sertoli cells

From the basal lamina to the lumen of the seminiferous tubule, there are Sertoli cells involved in mechanical support and nutrition of germ cells providing critical factors necessary for the successful progression of germ cells into spermatozoa, through the complex process of spermatogenesis (Griswold, 1998; Kerr, 1992; Silva and Carvalho, 2010) (see section 1.2.1.). Sertoli cells have receptors to testosterone and Follicle-stimulating hormone (FSH) (McLachlan et al., 2006). Testosterone has a negative feedback on the pituitary gland suppressing LH secretion (Damassa et al., 1976). Sertoli cells are also responsible to the maintenance of the integrity of the seminiferous epithelium by establishing tight junctions (blood-testis barrier) (Tsukita, Furuse and Itoh, 2001; Pelletier and Byers, 1992).

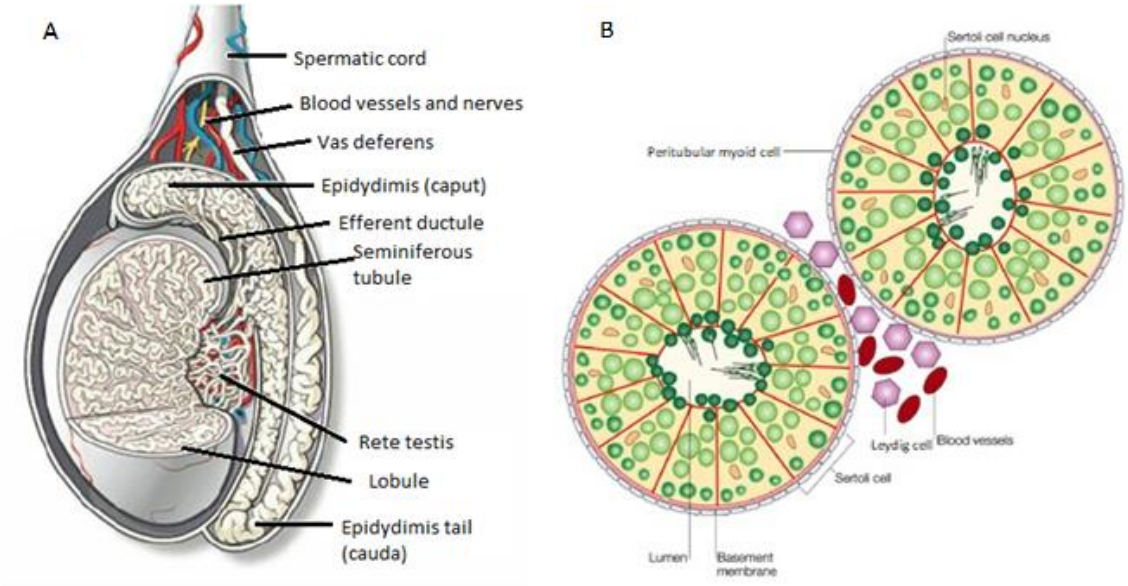

Figure 1 - Testicles (A) Cross-section showing the location of the seminiferous tubules, the vas deferens and the epididymis. Adapted from Desai et al., 2017. (B) Schematic cross-section of a testicular tubule, illustrating Sertoli cells, peritubular myoid cells and Leydig cells (in the interstitium). Retrieved from Cooke and Saunders, 2002.

1.2. Spermatozoa

Germ cells divide and differentiate forming the male gametes (spermatozoa), though spermatogenesis (Griswold, 1998; Kerr, 1992; Silva and Carvalho, 2010).

1.2.1. Spermatogenesis

In humans, the progression from spermatogonia to spermatozoa take approximately 64 days (Heller and Clermont, 1963). In the seminiferous tubules, we can find cells increasing the order of maturation: spermatogonia, spermatocytes (primary and secondary), spermatids and spermatozoa (Figure 2). Spermatogenesis is a process that occurs from the periphery to the lumen of the tubules and requires several hormones namely LH, FSH, testosterone and growth hormone (GH) (Sadler, 2011). In humans, spermatogenesis is divided into three different phases: mitotic phase, meiotic phase (I and II) and spermiogenesis (Kanakis and Goulis, 2015).

Approximately at the same time, primordial germ cells give rise to spermatogonia, diploid cells that can be of two types: spermatogonia type A, which divide by mitosis to form part of a continuous reserve of stem cells and spermatogonia of type B, which by mitosis give rise to primary spermatocytes, during approximately 22 days, followed by rapid termination of meiosis I and formation of secondary spermatocytes (Bras et al., 1996). During the second meiotic division, these cells begin to form haploid spermatids. The last stage of spermatogenesis, spermiogenesis, constitutes the spermatozoa formation from spermatids. Includes, for instance, the formation of the acrosome, which covers half of the nuclear surface and contains enzymes that help fertilization (Zaneveld and De Jonge, 2013) and when fully formed, spermatozoa are carried by the contractile elements present in the walls of the seminiferous tubules and the tubular fluid secreted by the Sertoli cells into the epididymis, where sperm motility is acquired (Haschek, Rousseaux and Wallig, 2009).

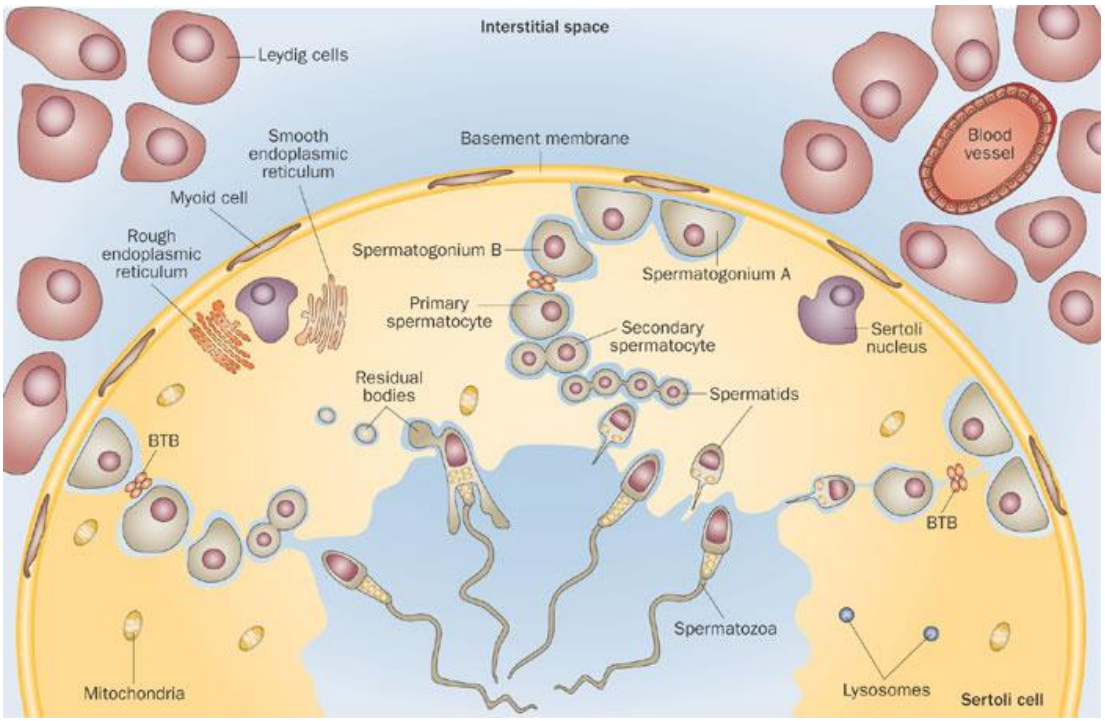

Figure 2 – Schematic illustration of spermatogenesis. Retrieved from Rato et al., 2012.

1.2.2. Spermatozoa structure

The spermatozoa may be divided into two regions: the head and the flagellum (Figure 3). The head is about 4,39 µm long (Bellastella et al., 2010) and compromises the nucleus, protected by the perinuclear theca (Terada et al., 2000), which contains the DNA (Deoxyribonucleic acid) condensing core and protamines, positively charged DNA proteins rich in arginines (Balhorn, 2007), responsible to enable nuclear hypercondensation and ensure the sperm genome remains inactive until it can be deposited inside an egg and reactivated (Brewer, Corzett and Balhorn, 2002). There are two types of protamines: protamine 1 (P1) and the family of protamine 2 (P2, P3 and P4); genes protamine mutations and changes in their expression are associated with male infertility (Oliva, 2006).

The acrosome is a Golgi-derived secretory vesicle that covers 47.5% of the head (Bellastella et al., 2010). This structure containing hydrolytic enzymes (as acrosin and hyaluronidase) that digest zona pellucida, helping the sperm penetrate the oocyte (Yoshinaga and Toshimori, 2003). Measuring acrosin activity is a suitable approach for

evaluating the fertilizing capacity of human spermatozoa, because men with leukospermia (presence of leukocytes in the ejaculate above the threshold value (WHO, 2010)), and abnormal semen parameters present low actin activity (Zalata et al., 2004). The hyaluronidase is responsible to enable acrosome-intact sperm to reach the zona pellucida by hyaluronan hydrolysis (Kimura et al., 2009).

The flagellum consists of four regions: connecting piece, mid-piece, principal piece and final piece (Inaba, 2003). The connecting piece contains a proximal centriole and distal centriole that gives rise to the axoneme, which extends throughout the length of the entire flagellum requiring proteins such as tubulin, nexin, and dynein for its formation (Jin, Wang and Fang, 2014). The mid-piece presents nine outers dense fibers (ODFs) and a mitochondrial sheath (MS) that encloses the ODFs and the axoneme (Olson and Sammons, 1980). Mitochondria are found only in the MS of the mid-piece. As in other cells, sperm mitochondria produce Adenosine-5-triphosphate (ATP) through aerobic respiration. The ODFs extend into the principal piece of the flagellum (Turner, 2005; Inaba, 2003). Between the end of the mid-piece and the beginning of the principal piece are the annulus (Turner, 2005). Into the principal piece, the MS terminates and two of the ODFs are replaced by two longitudinal columns of fibrous sheet (FS). The FS is restricted to the principal piece and has A-kinase anchoring proteins (AKAPs) like A-kinase anchor protein 4 (AKAP4). AKAPs tether cyclic AMP-dependent protein kinases and thereby localize phosphorylation of target proteins and initiation of signal-transduction processes triggered by cAMP (Cyclic adenosine monophosphate) (Miki et al., 2002; Lindemann and Lesich, 2010). The final piece is the short terminal portion of the flagellum and has only the axoneme surrounded by the plasma membrane (Turner, 2005).

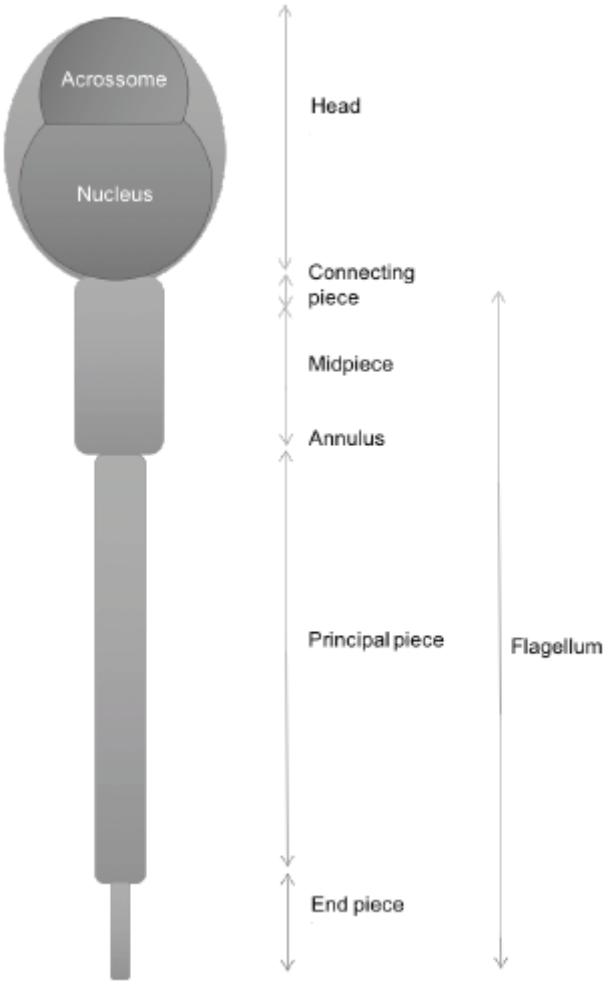

Figure 3 - Schematic representation of the spermatozoa. The head contains the acrosome and the nucleus. The flagellum is divided structurally into four areas: the connecting piece, mid-piece, principal piece and end-piece. Retrieved from Fardilha, Silva and Conde, 2015.

1.3. Epididymis

Although the human epididymis does not have defined sections in comparison to other primates (Cooper, 2012), this segmented organ can be divided into four main anatomical regions: initial segment (closest to the testis), caput (region between the initial segment and the corpus), corpus and cauda (closest with the vas deferens) (Martan, 1969; Ivell, 2007). The segments display distinct ions concentrations and differential expression of genes, essential to regulate sperm maturation. Spermatozoa need to undergo two

maturational processes and successive biochemical changes to become competent to fertilize the oocyte: maturation in the epididymis (male reproductive system) and capacitation (female reproductive system) (Bearer and Friend, 1990).

1.3.1. Epidydimal sperm maturation

After spermiation (release of immature sperm into the lumen of the seminiferous tubules), immature spermatozoa need to undergo a maturation process in the epididymis to become motile. Those include alterations in intracellular pH, calcium (Ca^{2+}) concentration and cAMP responsible for proteins phosphorylation (Huang and Vijayaraghavan, 2004). Spermatozoa are subject to morphological and biochemical changes such as post-translational protein modifications, cytoplasmic droplet (cytoplasmic excess) migration along the middle piece and a decrease of the head size (Fardilha, Silva and Conde, 2015; Cooper, 2011). As well, during epidydimal transit spermatozoa acquire new proteins like zona pellucida sperm-binding protein (ZP3), enzymes of the polyol pathway (HE5/CD52), type 5 glutathione peroxidase (GPX5) and sperm adhesion molecule 1 (PH-20) (Sullivan, Frenette and Girouard, 2007). These proteins are responsible for sperm maturation and they are present in the epididymosomes, membranous vesicles secreted in the intraluminal compartment of the epididymis (Saez, Frenette and Sullivan, 2003).

During the epididymal transit, the levels of Ca^{2+} decrease and the levels of intracellular pH and cAMP increase. It is known that cAMP is responsible for the activation of protein kinase A (PKA) and this leads the initiation and stimulation of spermatozoa motility (Chakrabarti et al., 2007).

Ser/Thr phosphatases play a key role in activation of sperm motility (Tash and Bracho, 1994; Ickowicz, Finkelstein and Breitbart, 2012). For instance, phosphoprotein phosphatase 1 catalytic subunit gamma 2 (PPP1CC2), a testis-enriched and sperm-specific PPP1 isoform, is a key player in sperm motility acquisition (Fardilha et al., 2011; Fardilha et al., 2013). In caput region, spermatozoa are immotile. Glycogen synthase kinase 3 (GSK3) phosphorylates PPP1R2 at Thr73 which inhibits the interaction between PPP1R2 and PPP1CC2 resulting in active PPP1CC2 (Silva, Freitas and Fardilha, 2014). Also,

spermatozoa at this stage have high cholesterol, non-phosphorylated sperm-egg fusion protein 1 (IZUMO1), low membrane fluidity and low levels of transfer ribonucleic acid (tRNA) fragments (Calvel, et al., 2010; Leahy and Gadella, 2015; Ellerman et al., 2010). Contrary, in the cauda region, PPP2CC2 is associated to PPP1R2 and consequently inactive (Somanath, Jack and Vijayaraghavan, 2004). Ca^{2+} influx activates sAC, which produces cAMP activating Rap guanine nucleotide exchange factor (RAPGEFs), which activates Protein kinase B (PKB), also know AKT, that phosphorylates GSK3. GSK3 inhibition leads to decrease Thr73 PPP1R2 phosphorylation. Consequently, PPP1R2 binds PPP1CC2. As well, a multimeric complex has been identified composed by PPP1CC2, sds22 (PPP1R7), actin and I3 (PPP1R11), where PPP1CC2 was inactive. Thus, PPP1 activity is inhibited and ser/thr phosphorylation of key residues increases leading to motile spermatozoa (Korrodi-Gregório et al., 2013). Cauda spermatozoa have high phospholipid content, extensively phosphorylated IZUMO1, high membrane fluidity and high levels of tRNA fragments (Calvel et al., 2010; Miranda et al., 2009; Leahy and Gadella, 2015). Mature spermatozoa are stored in the cauda region of the epididymis (Fardilha, Silva and Conde, 2015). Other functions like spermatozoa immunoprotection also occur in the epididymis (Marchiani et al., 2017).

1.4. Sperm capacitation

Sperm capacitation is a physiological process that spermatozoa must undergo to acquire fertilization capability, being necessary structural and biochemical changes that occur in the spermatozoa during passage through the female reproductive tract (López-González et al., 2014). Spermatozoa is a differentiated cell devoid of transcription and translation machinery and, after ejaculation, these cells are deposited in the vagina, an environment with hostile conditions like acidic internal pH (Qi et al., 2007) and a high concentration of progesterone and albumin (Abou-haila and Tulsiani, 2009). Progesterone is a messenger responsible for hyperactivation of the motility, cross-reaction and chemotaxis of sperm with the effect of Ca^{2+} induction (Lishko, Botchkina and Kirichok, 2011; Strünker et al., 2011).

After passage through the cervix, spermatozoa arrive at the uterus undergoing several structural and biochemical changes, such as the loss of plasma membrane cholesterol (Martínez and Morros, 1996). After this, proteins secreted by oviductal tissue have been associated with capacity-related molecular events, such as tyrosine (Tyr) phosphorylation of sperm proteins and the ability to respond to acrosome reaction (Zumoffen et al., 2010). Also, these proteins bind to the spermatozoa and protect them from oxidative damages induced by reactive oxygen species (ROS) and stimulate sperm motility (Huang et al., 2013). For instance, sperm fucosyltransferase-5 (sFUT5) is a membrane carbohydrate-binding protein on human spermatozoa involved in spermatozoa–oviduct interaction that contributes to the success of fertilization (Huang et al., 2015). A recent study, showed that peroxiredoxins (antioxidant enzymes) are necessary to control the concentration of ROS generated during capacitation and infertile men have lower levels of peroxiredoxins (Lee et al., 2017). Other process occurs during capacitation is the acquisition of hyper-activated motility that results of phospholipase D-dependent actin polymerization, allowing sperm to penetrate with greater flexibility the mucus present in the fallopian tubes (Itach et al., 2012). As well, ionic alterations occur during sperm capacitation: the increasing of intracellular bicarbonate (HCO_3^-) and Ca^{2+} concentration, through voltage-dependent channels and cation channels of sperm (CatSper), leads to the activation of soluble adenylyl cyclase (sAC), which in turn promotes the increase of cAMP activating PKA leading to the production of superoxide ($O_2^{\cdot-}$), causing the increase of phosphorylated proteins (Chung et al., 2017; Cruz et al., 2014; Qi et al., 2007; Fardilha, Silva and Conde, 2015; Alasmari et al., 2013). HCO_3^- influx also leads to the increasing of intracellular pH, causing of the sperm plasma membrane hyperpolarization (López-González et al., 2014; Leemans et al., 2016).

1.5. Signaling pathways in spermatozoa

1.5.1. sAC/cAMP/PKA

The sAC/cAMP/PKA pathway is responsible for protein phosphorylation in the spermatozoa (Abou-haila and Tulsiani, 2009). cAMP has been reported to be essential for events occurring during capacitation, including activation of motility, hyperactivation and acrosome reaction (Buffone et al., 2014).

sAC plays a critical role in cAMP signaling in spermatozoa: the increase of HCO_3^- and Ca^{2+} activate the sAC, which is responsible to convert AMP in cAMP (Xie et al., 2006). PKA is not essential for spermatogenesis and spermiogenesis, however, PKA does play a critical role in sperm capacitation and motility that are required for fertilization (Burton and McKnight, 2007). PKA structure consists of two regulatory subunits (RIα and RIIα) and two catalytic subunits (Cα1 and Cα2). RIα is expressed throughout spermatogenesis, while RIIα only appears at the late stages in spermatogenesis (Burton and McKnight, 2007). The increase of cAMP leads to the activation of PKA which causes the dissociation of active sperm catalytic subunit (Cα2). Cα2 phosphorylates downstream targets that lead sperm capacitation by increasing motility, Ca^{2+} entry and tyr phosphorylation (Burton and McKnight, 2007; Kaupp and Strünker, 2017). PKA inhibits the production of cAMP by directly or indirectly inhibiting sAC activity. PKA activation also leads to an increase in actin polymerization, a process responsible for the development of hyperactivated motility (Ickowicz, Finkelstein and Breitbart, 2012). The process of activation/inhibition of this pathway is obtained by negative feedback, the action of phosphodiesterase (PDE) and activation of specific ser/tyr phosphatases (Fardilha, Silva and Conde, 2015). With the increase of the Ca^{2+} in the intracellular fluid, this ion binds to the calmodulin, multifunctional Ca^{2+} binding protein, forming a complex that is responsible for the activation of PDE. PDE hydrolyses cAMP in AMP, inhibiting sAC/cAMP/PKA pathway. Thereby, Ca^{2+} has a dual function: it is a secondary messenger in the activation of sAC and participates in regulation when binding to calmodulin, inhibiting sAC/cAMP/PKA pathway.

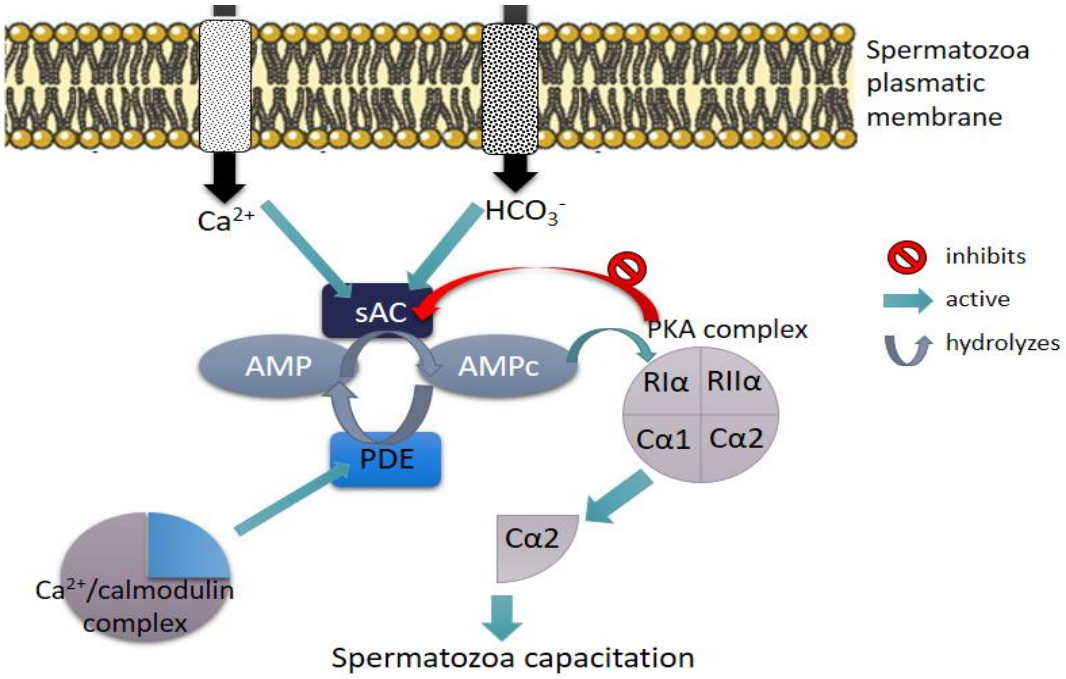

Figure 4 - Schematic representation of sAC/cAMP/PKA pathway in sperm. The activation of this pathway is obtained by the increase of HCO_3^- and Ca^{2+}. These ions activate the sAC, which is responsible to convert AMP in cAMP. The increase of cAMP leads to the activation of PKA which causes the dissociation of Cα2, leading sperm capacitation. The inhibition of this pathway is obtained by sAC inhibition by PKA complex and the increase of calmodulin/Ca^{2+} complex that is responsible for the activation of PDE. PDE hydrolyses cAMP in AMP. sAC: soluble adenylyl cyclase; AMP: adenosine monophosphate; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; Cα2: active sperm catalytic subunit; PDE: phosphodiesterase. Adapted from Fardilha, Silva and Conde, 2015; Buffone et al., 2014.

1.5.2. Phospholipase C

Phospholipase C/PI3/Ca^{2+} is signal pathway essential for actin dispersion and acrosome reaction (Breitbart, 2003). This pathway is initiated when progesterone and ZP3 bound to receptors localized in the anterior region of the spermatozoa head: inhibitory G protein-coupled receptors (Gi) and tyr kinase receptors (Schwartz et al., 2016).

Phospholipase C isoforms include six families of enzymes (PLC-β, γ, δ, ε, ζ and η), based on their biochemical proprieties, that play a key role in a wide array of intracellular signaling pathways (Béziau et al., 2015). PLC-β is associated to G protein-coupled receptors (McCudden et al., 2005). In turn, PLC-γ is associated with tyr kinase receptors

(Nishibe et al., 1990; Nakamura and Fukami, 2017). These two isoforms of the phospholipase C hydrolyse phosphotidylinositol 4,5-biphosphate (PIP2) to phosphoinositol 3-phosphate (PI3) and diacylglycerol (DAG) (Breitbart, 2003). PI3 induce the release of Ca^{2+} from internal Ca^{2+}store (acrosome) to the cytosol (Walensky and Snyder, 1995). DAG activates the protein kinase C (PKC) to open Ca^{2+}channels in the plasma membrane (Breitbart, 2002; Fukami et al., 2010). Progesterone also induces Ca^{2+} influx into spermatozoa, essential for sperm hyperactivation, acrosome reaction and chemotaxis towards the egg (Lishko, Botchkina and Kirichok, 2011).

The PIP2 hydrolysis, the depletion of Ca^{2+} in the acrosome and the increase of Ca^{2+} concentration in the cytosol leads to actin hydrolysis (Breitbart and Finkelstein, 2015). The polymerization of the actin allows the approach of the acrossomatic and plasmatic membranes and their fusion, which is essential for the acrosome reaction (Fardilha, Silva and Conde, 2015). The Ca^{2+} depletion in the acrosomic zone stimulates the action of SOC allowing the Ca^{2+} influx of the extracellular medium to the cytosol (Breitbart, 2002).

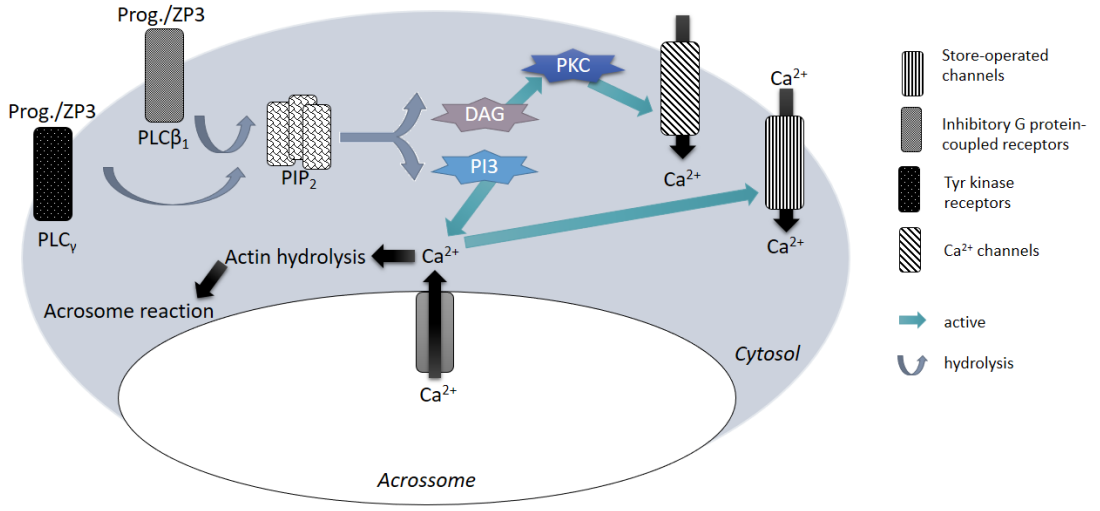

Figure 5 - Schematic representation of Phospholipase C pathway in sperm. Progesterone and ZP3 bound to Gi and tyr kinase receptors, which are associated PLC-β1 and PLC-γ, respectively. PLC isoforms hydrolyse PIP2 to DAG and PI3. DAG activates PKC that activates Ca^{2+} channels. PI3 induces Ca^{2+} release from acrosome to the cytosol, leading actin hydrolysis and consequently acrosome reaction. ZP3: zona pellucida sperm-binding protein 3; Gi: inhibitory G protein-coupled receptors; tyr: tyrosine; PLC-β1: phospholipase C β1 isoform; PLC-γ: phospholipase C γ isoform; PIP2: phosphotidylinositol 4,5-biphosphate; DAG: diacylglycerol; PI3: phosphoinositol 3-phosphate. PKC: protein kinase C. Adapted from Fardilha, Silva and Conde, 2015; Breitbart, 2003.

1.5.3. ROS/MAPK pathway

ROS such as O^{2-}, hydrogen peroxide (H_2O_2) and nitric oxide (NO^-) may have contradictory effects: high levels of ROS, mostly H_2O_2, have deleterious effects on spermatozoa and may cause irreversible damage in sperm DNA (de Lamirande and Gagnon, 1995). However, in low and controlled levels, ROS are essential for spermatozoa capacitation (De Lamirande, Leclerc and Gagnon, 1997) modulating signals pathways, like mitogen-activated protein kinase (MAPK) pathway (Bailey, 2010).

The signal transduction is initiated by Shc (Src homology collagen) which will be phosphorylated and after this, Shc active Grb2 (Growth factor receptor-bound protein 2). Shc and Grb2 are examples of adapter proteins (Fardilha, Silva and Conde, 2015). Grb2 activate Sos (son of sevenless) and Sos activate Ras (family of GTP-binding proteins), responsible to activate ERK cascade that it plays a role upstream of protein tyr phosphorylation (O'flaherty, de Lamirande and Gagnon, 2006). H_2O_2, a major ROS in sperm, activates protein kinase C, which will activate rapidly accelerated fibrosarcoma (Raf). PI3K phosphorylates PDK1 which phosphorylates Akt. Akt stimulates NO^- synthetase resulting in increased concentration of this ion which consequently activates Ras (Aitken, 2017; Fardilha, Silva and Conde, 2015). Components of the extracellular signal-regulated kinase (ERK) family of MAPK pathways are present in spermatozoa (Urnerand and Sakkas, 2003). Raf phosphorylates MEK, specific kinases for the thre-glu-tyre module, present in ser/thr-specific kinases, like ERK1 and ERK2: they are active during capacitation by enhanced phosphorylation and, they blocked both protein tyr phosphorylation and the ability of the sperm to acrosome-reaction (Thundathil, De Lamirande and Gagnon, 2002).

Capacitation is also associated with the phosphorylation of MEK and MEK-like proteins (O'Flaherty, de Lamirande and Gagnon, 2005). The addition of H_2O_2 increase the levels of P-MEK-like proteins during capacitation and MEK inhibitors, like PD98059 and U126, are responsible for blocking the rise in P-MEK-like proteins and sperm capacitation (O'Flaherty, de Lamirande and Gagnon, 2006). The capacitation-related increase in P-MEK-like proteins induced by FCSu is modulated by PKA and PKC and it was hypothesized that these P-MEK-like proteins probably represent an intermediary step

between the early events and the late PKA-dependent Tyr phosphorylation associated with capacitation (O'flaherty, de Lamirande and Gagnon, 2006). Therefore, ROS seem to regulate many of the events related to sperm capacitation.

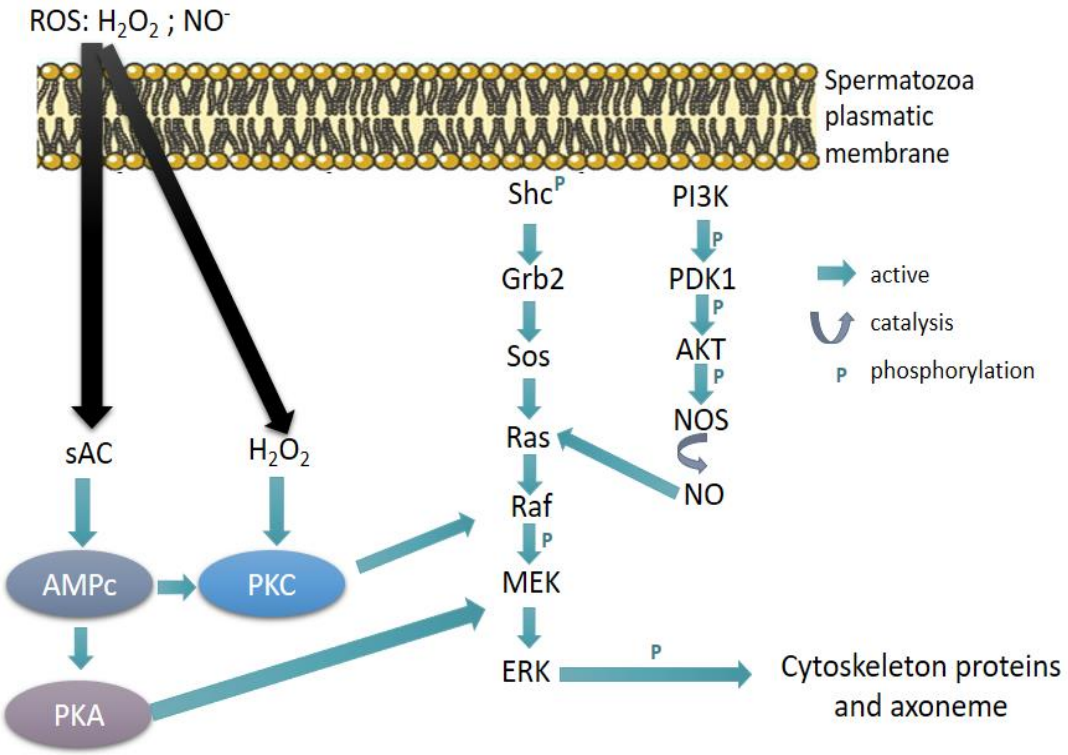

Figure 6 - Schematic representation of ROS/MAPK pathway in sperm. ROS activate sAC. sAC promotes the activation of the PKA that activate MEK. H_2o_2 activates PKC which in turn activates Raf. PI3K phosphorylates PDK1 which in turn, phosphorylates AKT. AKT stimulates NO synthesis activating Ras. ROS: Reactive oxygen species; sAC: soluble adenylyl cyclase; PKA: protein kinase A; MEK: mitogen activated kinase; H_2o_2: hydrogen peroxide; PKC: protein kinase C; Shc: Src homology collagen; Grb2: Growth factor receptor-bound protein 2; Sos: son of sevenless; ERK: extracellelar signal-regulated kinases; Raf: rapidly accelerated fibrosarcoma; PI3K: phosphatidylinositide 3-kinase; PDK1: phosphoinositide-dependent kinase-1; AKT: protein kinase B; NOS: nitric oxide synthase; NO: nitric oxide; Ras: rat sarcoma protein. cAMP: cyclic adenosine monophosphate. Adapted from O'flaherty, de Lamirande and Gagnon, 2006; Fardilha, Silva and Conde, 2015.

1.6. Infertility

According to the World Health Organization, infertility is the inability to achieve a pregnancy after twelve months or more keeping unprotected regular intercourse (Zegers-Hochschild et al., 2006). About 25% of couples in reproductive age have fertility-related problems, with an estimated 15% seeking medical treatment for infertility (Kassebaum et al., 2014).

The male factor is involved in 50% of the cases of conjugal infertility, being exclusively responsible for approximately 20% of the cases (Zegers-Hochschild et al., 2009). This percentage has been rising in recent years due to causes as diverse as the postponement of maternity, increased prevalence of sexually transmitted infections, sedentary lifestyle, obesity, tobacco and alcohol consumption and pollution (Jungwirth et al., 2012; Jefferys, Siassakos and Wardle, 2012).

The evaluation of the infertile man should be performed in stages, beginning with the clinical history, physical examination, the cytochemical study of sperm and careful laboratory tests, in the attempt to classify the cause of male infertility.

In Portugal, primary infertility is about 2,1% and secondary infertility 9,2% (Mascarenhas et al, 2012). In Portugal, women age at the first child is increasing: in 1960 the mean age was 25,0 years comparably to 2016 the mean age was 30,3 years (Pordata, 2016).

In 2014, a fertility survey performed a Portuguese woman with ages between 18 and 49 years and Portuguese men with ages between 18 and 54 years, showed that the financial costs associated with children and the difficulty in finding a job are the main reasons to postpone paternity (INE, 2014). In other survey, it was concluded that men with highest academic qualifications postponed further paternity in comparison to men with lower qualification. In addition, men (53%) consider having children more important for their personal fulfilment in comparison to women (46%) (FFMS, 2016).

1.6.1. Impact of age on male infertility

Most of age-related fertility studies focus on female age. However, recent studies have shown that advancing male age is associated with changes in semen parameters, compromising fertility (Harris et al., 2011; Sloter et al., 2006).

The cause of the age-related decline in semen parameters quality has not been clearly defined, however, there are latent age-related diseases, like obesity and genital tract infections, that increase, for instance, the incidence of low progressively motile spermatozoa (Hammoud et al., 2008). The significance of the observed age-related changes in semen parameters remains a controversy because some studies cannot account the relationship between paternal age and reduced pregnancy rate (Koh et al., 2013; Belloc et al., 2014; Keel, 2006).

Several studies indicate that increased male age is associated with a decline in semen volume, sperm motility, and normal sperm morphology but not with sperm concentration (Kidd, Eskenazi and Wyrobek, 2001; Mukhopadhyay et al., 2010).

In contrast, some studies detected that older men have also a significant decrease in sperm concentration (Maya, Berdugo and Jaramillo, 2009), alpha-glucosidase and fructose seminal levels (Molina et al., 2010), and an increase of ROS levels compared with younger men (Cocuzza et al., 2008).

There is also a relationship between sperm DNA quality and age: several studies demonstrated that DNA fragmentation increasing with age, probably caused by defective sperm chromatin packaging, disordered apoptosis and oxidative stress (Agarwal and Said, 2003; Moskovtsev, Willis and Mullen, 2006; Brahem et al., 2011; Evenson et al., 2014). As well, in recent study oxidative stress markers in human semen were evaluated (Koh, Sanders and Burton, 2016). It was demonstrated that older males (40 years of age or above) exhibited higher levels of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and a decrease in sperm concentration and motility. This study suggests that 8-OHdG may be an important biomarker between male age and fertility.

Additionally, advanced paternal age has been associated with lower pregnancy rates, higher risk of pregnancy loss and with childhood health, particularly with higher incidence of congenital birth defects and disorders like achondroplasia, autism, schizophrenia, trisomy and some types of cancers (Sharma et al., 2015).

All these studies showed that the age of male partner has a deleterious impact sperm parameters and DNA quality, which may, in part, contribute to negative reproductive outcomes.

1.7. Objective

The studies available regarding the effects of age on male fertility are scarce. Most of those studies are only focused on basic semen parameters and reproductive outcomes. Despite the scientific consensus that male age is an important factor, very little is understood about the molecular mechanisms underlying the relationships between male age and reduced fertility. **The main objective of this study is to evaluate the impact of aging on human spermatozoa signaling proteins**.

2. Material and Methods

2.1. Study Overview

Experimental procedures were performed in Signal Transduction Laboratory, iBiMED Institute for Biomedicine, University of Aveiro (Aveiro, Portugal) and in Unidade de Procriação Medicamente Assistida, COGE – Clínica Obstétrica e Ginecológica de Espinho (Espinho, Portugal).

2.2. Human samples collection

In this study 31 men undergoing Artificial Insemination (AI), *In Vitro* Fertilization and Intracytoplasmic Sperm Injection (ICSI) treatments or semen analysis at COGE (Clínica Obstétrica e Ginecológica de Espinho) were included. All men signed a written consent authorizing the use of the samples for research purposes (Appendix 1).

2.3. Basic Semen Analysis

Ejaculated human semen samples from donors were collected by masturbation into a sterile container at COGE. Basic semen analysis was conducted in accordance with World Health Organization (WHO, 2010) guidelines. Basic semen analysis was performed using an Eclipse E400 (Nikon) microscope.

2.3.1. Macroscopy parameters evaluation

At the macroscopic level, the following parameters were evaluated: colour, smell, volume, liquefaction, pH and viscosity. The colour and smell were analysed directly by the observer. The volume was measured directly by aspirating the sample from the container into a graduated pipette or 1ml syringe (samples with a volume less than 1ml). After 60 minutes, liquefaction was analysed and pH was measured using pH paper in the range 6,0 to 10,0. The viscosity of the sample was estimated by gently aspirating it into a wide-bore plastic disposable pipette, allowing the semen to drop by gravity.

2.3.2. Microscopy parameters evaluation

At the microscopic level, the following parameters were evaluated: motility, concentration, vitality, morphology and the presence of leukocytes.

2.3.2.1. Motility

Sperm motility was measured as soon as possible after liquefaction of the sample using Nikon eclipse E400 microscope. Firstly, was prepared a slide with 20ul of fresh sample and using a manual cell counter, two counts (5 different fields) with more than 100 spermatozoa were performed and sperm classified in 4 categories (type a, b, c (*in situ*) and d).

2.3.2.2. Concentration

For the evaluation of the concentration, a preliminary observation was performed on a slide and this will allow calculating which dilution more suitable to use. Generally, 1:20 dilution (20 ul of semen with 380ul of distilled water) was used. After this, 10ul of the diluted sample was placed in Neubauer hemocytometer (two separate counting chambers) and waited 15 minutes, to allow sedimentation of the spermatozoa. Using Nikon eclipse E400 microscope, the spermatozoa with head and tail of 5 squares (central, upper left, upper right, lower left and lower right) was counted.

2.3.2.3. Vitality

For vitality test, firstly was prepared a 1:1 diluted sample (40ul eosin + 40ul semen). Using Nikon eclipse E400 microscope (phase contrast) was performed 100 counts of spermatozoa with different colours of cell membrane: red/pink (spermatozoa with damaged cell membrane) and greenish (spermatozoa with integrate cell membrane), a total of 100 spermatozoa.

2.3.2.4. Morphology

To determinate the sperm morphology was prepared a smear of semen on two slides. After air-drying, the slides were fixed and stained (Papanicolaou and Shorr staining). After that, the slides were examined with bright field optics at 1000x magnification with oil immersion, using Nikon eclipse E400. Approximately 200 spermatozoa were assessed and classified, according to the strict criteria of Kruger. Using a cell manual counter were categorized and counted normal spermatozoa, spermatozoa with head defects, spermatozoa with neck and midpiece defects, spermatozoa with tail defects and spermatozoa with excess residual cytoplasm.

2.3.2.5. Leukocytes

The leukocytes were evaluated in a semen smear fixed and stained with the Papanicolaou and Shorr procedures. Using Nikon eclipse E400, the slides were observed with bright field optics at 1000x magnification with oil immersion. All classes of human leukocytes express a specific antigen (CD45) that can be detected with an appropriate monoclonal antibody, to allow detection of different types of leukocytes, such as macrophages, monocytes, neutrophils, B-cells or T-cells.

2.3.3. Semen Processing – Density gradients

The ejaculate contains a mixture of motile, immobile, dead, and possibly agglutinated spermatozoa, in addition to a set of debris (germ cells, exfoliated cells of the male tract, leukocytes and other amorphous material) along with the seminal fluid. Seminal fluid may be toxic to spermatozoa if their contact is delayed, so treatment should be performed poorly if the sperm is liquefied. Density gradients technique is based on the different density presented by spermatozoa. The gradients establish substrates of different densities so that the denser spermatozoa (which are the ones with the best morphology and motility) will go to the bottom of the tube after centrifugation, forming a pellet. This technique is used for Assisted Reproduction Technology (ART).

2.3.3.1. Semen Preparation by density gradients

After liquefaction, the semen was centrifuged (Labofuge 400, Heraeus) with gradients SupraSperm® (Origio, Denmark) at 55% and 80%, for 20 minutes at 300G. The supernatant was carefully aspirated and the sperm pellet resuspended and washed with Sperm Preparation Medium® (Origio, Denmark) for 10 minutes at 300G. The supernatant was again removed and Sperm Preparation Medium® (Origio, Denmark) was carefully added above the pellet and incubated at 37% at an angle of 45º (Swim-up technique).

2.4. Semen Cryopreservation

After Swim-up technique, Sperm Freezing Medium® (Origio, Denmark) was added by dropwise to the sample in 1:1 ratio. After 10-20 minutes at room temperature, the samples were carefully mixed and placed in horizontal position in liquid nitrogen vapor. After 20-30 minutes, the samples were emerged in liquid nitrogen (-196 ºC) and stored in an appropriate canister.

2.5. Spermatozoa Protein Extracts

Spermatozoa cells were incubated with 1X PathScan Sandwich ELISA Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with 1 mM PMSF for 5 minutes on ice. Then the lysed cells were centrifuged at 14000 g at 4ºC for 10 minutes and the supernatant was transferred to a new tube (cell lysate).

2.6. Protein Quantification - Bicinchoninic Acid Assay

Extracts were mass normalized using the bicinchoninic acid (BCA) assay (Fisher Scientific, Loures, Portugal). Sample was prepared to be assayed with 3 uL of each sample plus 22 uL of the lysis buffer. Standard protein concentrations were prepared as described in Table 1. Samples and standards were prepared in duplicate. The bovine serum albumin (BSA) stock solution used had a concentration of 2 mg/ml. The Working Reagent (WR) was prepared by mixing BCA reagent A with BCA reagent B in the proportion of 50:1.

Then, 200 µl of WR was added to each well (standards and samples) and the plate was incubated at 37 ºC for 30 min. Once the tubes cooled to RT the absorbance was measured at 562 nm using an Infinite® 200 PRO (Tecan, Switzerland). A standard curve was obtained by plotting BSA standard absorbance vs BSA concentration, and used to determine the total protein concentration of each sample.

Table 1 - Standards for BCA assay.

Standards	BSA (µl)	1% SDS (µl)	Protein (µg)
P_0	-	25	0
P_1	0.5	24.5	1
P_2	1	24	2
P_3	2.5	22.5	5
P_4	5	20	10
P_5	10	15	20

2.7. Antibody Array - PathScan® Intracellular Signaling Array

Antibody-based arrays were carried out using the PathScan® Intracellular Signaling Array Kit (#7744, Cell Signaling Technology, Danvers, MA, USA) to determine the expression patterns of 18 well-characterized signaling molecules when phosphorylated or cleaved, in 31 semen samples obtained from a randomized group of donors. Each cell extract was diluted to 0,2 ug/ul and applied to its own multiplexed array according to the manufacturer's instructions. Fluorescence readouts from the arrays were captured digitally using LI-COR® Biosciences Odyssey® imaging system (LI-COR® Biosciences, Nebraska, USA). Pixel intensity was quantified using Odyssey software. The intensity from the negative control within each array was subtracted from all signals, and all data from each array were normalized to the internal positive control within each array.

2.8. Statistical analysis

Statistical analysis was conducted using the IBM SPSS Statistics Software 22. First, a descriptive analysis to each quantitative parameter analyzed was performed. Then, the Pearson correlation coefficient, r, or the Spearman's rho correlation coefficient, rs, (a nonparametric correlation method) were analyzed to determine the relationship between variables. The significance level was set at 0,05.

3. Results

The study included 31 semen samples, obtained from male patients that underwent basic semen analyses at COGE, between March and June 2017. From those, 39% (12 patients) were submitted to ART, namely AI (4 patients), IVF (7 patients) and ICSI (1 patient). The cycles were performed with ejaculated spermatozoa. No pregnancies were achieved. The mean age of men included in this study was 36,8±6,9(SD) years. Data concerning smoking habits was collected (Table 2). From the 31 men included in this study, 5 were smokers. Given the reduced number of smokers in the study sample no further analysis were performed with this parameter.

According to the WHO's guidelines, basic semen parameters were analyzed and are presented in Table 2. The levels of signaling molecules (in distinct activation states) were determined with the PathScan Intracellular Signaling Array®, which includes antibodies for phosphorylated or cleaved signaling proteins (Table 3).

Initially, to evaluate the relationship between the results obtained from the seminal parameters analysis and patients' age, a Spearman's correlation test was performed (Table 4, Appendix 2). Next, the association between the expression patterns of the 18 signaling molecules and age was evaluated using Pearson correlation tests (Table 5).

The results indicated that only two basic semen parameters were significantly correlated with age – midpiece defects and excess of residual cytoplasm. Concerning sperm morphology, the percentage of midpiece defects showed a negative correlation with age (Spearman correlation coefficient=-0.43; p=0.012) Contrarily, the results showed a positive association between the excess of residual cytoplasm and age (Spearman correlation coefficient=0.37; p=0.034) (Table 4).

Furthermore, the results indicated that the levels of two phosphoproteins, p70 S6 kinase (Thr389) (Pearson correlation coefficient= -0,37; p= 0,047) and PRAS40 (Thr246) (Pearson correlation coefficient=0,54; p=0,002) showed a negative correlation with age (Table 5 and Figure 7).

Table 2 - Basic semen parameters of 31 patients providing semen samples for ART treatments or semen analysis. FIV, Fertilization In-Vitro; AI, Artificial Insemination; ICSI, Intracytoplasmic Sperm Injection.

CODE	Technic	Age (years)	Smoking habits	Sexual abstinence (days)	Volume (mL)	pH	Viscosity	Concentration (x10^6/mL)	No. Spermatozoa (x10^6)	Immotile (%)	Non-progressive (type c) (%)	Progressive (type b) (%)	Fast progressive (type a) (%)	Vitality (%)	Normal morphology (%)	Head defects (%)	Midpiece defects (%)	Tail defects (%)	ERC (%)	Leukocytes (x10^6/mL)	TZI
25994	Spermogram	29	No	3	2,1	8,1	High	72	151,2	129,2	12,0	25,6	15,4	54	0	98	39	19	11	72	1,67
25204	FIV	36	No	2	3,9	8,1	Normal	60	234,0	185,7	10,3	35,7	22,2	67	5	95	16	2	2	120	1,15
V1000	Spermogram	26	No	3	6,1	8,5	High	145	884,5	89,6	0,4	3,3	6,7	80	9	91	13	5	3	0	1,12
26052	Spermogram	43	No	3	3,5	8,3	High	15	52,5	58,0	30,9	8,8	2,2	33	0	99	20	7	8	30	1,34
25646	FIV	46	No	3	5,0	7,9	Normal	212	1060,0	91,9	0,7	3,0	4,3	73	4	96	13	6	6	0	1,21
25796	Spermogram	39	No	3	8,0	8,1	Normal	33	264,0	70,6	2,7	20,1	6,7	71	5	94	23	2	3	0	1,22
24882	AI	37	No	3	3,4	8,7	Normal	57	193,8	71,3	7,7	17,7	3,3	65	4	96	7	7	2	0	1,12
19108	Spermogram	18	No	3	0,5	8,7	High	40	20,0	19,4	29,1	44,7	6,8	57	5	95	33	4	4	0	1,36
24813	FIV	38	No	3	4,5	8,3	High	92	414,0	84,3	2,6	8,4	4,7	61	5	95	10	13	8	0	1,26
25586	FIV	38	No	3	1,6	8,1	High	114	182,4	71,4	7,4	7,4	13,7	66	5	95	24	1	0	0	1,20
V1100	Spermogram	31	Yes (12 p/day)	5	1,3	8,3	Lower	147	191,1	66,1	4,8	17,6	11,4	66	14	81	10	13	5	0	1,09
26283	Spermogram	33	No	6	6,6	8,1	Normal	113	745,8	90,3	2,3	6,5	0,8	66	3	97	17	3	1	113	1,18
25885	AI	46	No	3	4,3	8,3	Normal	103	442,9	83,9	2,3	9,9	4,0	63	13	88	11	10	3	103	1,12
26342	Spermogram	40	No	3	4,1	8,5	Normal	61	250,1	73,1	5,0	19,3	2,6	73	2	98	23	5	3	61	1,29
26382	Spermogram	38	No	2	4,1	8,1	Lower	86	352,6	80,2	1,8	13,6	4,3	69	17	83	20	3	0	0	1,06
V1200	Spermogram	29	Yes (16 p/day)	3	2,5	9,0	Normal	93	232,5	78,4	3,7	2,7	15,2	60	10	90	33	8	3	0	1,34
26357	ICSI	45	No	3	3,5	8,5	Normal	22	77,0	48,4	11,3	28,3	11,9	62	2	98	14	5	19	0	1,36
26511	Spermogram	44	Yes (e-cigarette)	2	1,3	8,3	Normal	97	126,1	62,4	7,4	8,9	21,3	68	9	90	12	5	4	0	1,11
26457	Spermogram	36	No	3/4	2,6	8,1	Normal	115	299,0	81,0	4,9	7,3	6,8	59	9	91	29	6	3	230	1,29
24832	AI	34	No	3	1,5	8,7	High	105	157,5	66,6	3,4	18,6	11,4	65	5	95	18	2	1	0	1,16
26531	Spermogram	45	No	5	4,3	7,9	Normal	93	399,9	80,0	1,8	9,4	8,8	79	18	68	28	4	4	0	1,04

CODE	Technic	Age (years)	Smoking habits	Sexual abstinence (days)	Volume (mL)	pH	Viscosity	Concentration (x10^6/mL)	No. Spermatozoa (x10^6)	Immotile (%)	Non-progressive (type c) (%)	Progressive (type b) (%)	Fast progressive (type a) (%)	Vitality (%)	Normal morphology (%)	Head defects (%)	Midpiece defects (%)	Tail defects (%)	ERC (%)	Leukocytes (x10^6/mL)	TZI
26432	Spermogram	32	No	3	3,1	8,1	High	114	353,4	82,3	2,1	10,7	4,9	62	4	94	40	6	1	0	1,41
26493	Spermogram	40	No	4	4,8	7,9	Normal	110	528,0	87,7	1,3	8,8	2,2	61	10	90	34	10	7	110	1,41
26619	Spermogram	42	No	4	0,7	9,0	High	4	2,8	5,6	48,2	44,2	2,0	30	2	98	28	3	5	0	1,34
25823	Spermogram	30	No	3	2,2	8,1	High	35	77,0	56,6	19,1	14,0	10,3	51	3	97	36	5	5	114	1,43
23408	FIV	35	No	3	4,4	8,1	High	55	242,0	69,9	3,5	14,5	12,1	75	5	95	32	2	2	0	1,31
26846	Spermogram	44	No	4	1,9	8,1	Normal	57	108,3	59,4	13,7	24,7	2,2	54	4	96	10	5	5	228	1,16
12520	FIV	46	No	3	1,2	8,1	Normal	92	110,4	56,8	3,1	23,7	16,5	68	8	92	8	3	6	0	1,09
19317	FIV	41	Yes (10 p/day)	3	4,4	8,5	Normal	49	215,6	75,2	3,8	17,8	3,1	60	7	91	17	3	1	4,4	1,12
V1234	Spermogram	24	Yes (15 p/day)	1	6,0	8,3	Lower	27	162,0	63,8	7,1	25,6	3,5	83	8	92	11	3	3	0	1,09
23407	AI	35	No	3	5,2	8,1	High	62	322,4	80,1	1,2	12,4	6,2	65	9	90	28	4	11	0	1,33

Table 3 - Expression patterns of 18 signaling molecules when phosphorylated or cleaved (PathScan® Intracellular Signaling Array) of 31 patients providing semen samples for ART treatments or semen analysis.

CODE	ERK1/2 (T202/Y204)	Stat1 (Y701)	Stat3 (Y705)	Akt (T308)	Akt (S473)	AMPKa (T172)	S6 Ribossomal Protein (S235/236)	mTOR (S2448)	HSP27 (S78)	Bad (S112)	p70 S6 kinase (T389)	PRAS40 (T246)	p53 (S15)	p38 (T180/Y18)	SAPK/JNK (T183/Y185)	PARP Asp214 Cleavage	Caspase-3 Asp175 Cleavage	GSK-3b (S9)
12520	0,128	0,138	0,318	0,103	0,118	0,328	0,068	0,138	0,093	0,123	0,113	0,083	0,053	0,068	0,128	0,083	0,133	0,303
19317	0,117	0,117	0,177	0,082	0,127	0,187	0,062	0,127	0,142	0,262	0,067	0,122	0,107	0,067	0,137	0,067	0,177	0,237
25204	0,055	0,065	0,115	0,035	0,055	0,120	0,020	0,050	0,040	0,060	0,040	0,035	0,020	0,030	0,055	0,030	0,050	0,130
24813	0,085	0,090	0,150	0,055	0,085	0,105	0,050	0,060	0,045	0,100	0,095	0,060	0,050	0,050	0,095	0,060	0,070	0,140
25646	0,122	0,152	0,162	0,077	0,092	0,167	0,057	0,087	0,062	0,107	0,092	0,062	0,047	0,047	0,122	0,052	0,077	0,207
25796	0,093	0,093	0,193	0,063	0,083	0,143	0,043	0,063	0,053	0,088	0,098	0,048	0,033	0,038	0,103	0,048	0,068	0,178
25885	0,125	0,105	0,170	0,070	0,100	0,145	0,050	0,080	0,050	0,100	0,090	0,055	0,050	0,055	0,105	0,070	0,075	0,210
26052	0,083	0,068	0,108	0,083	0,063	0,043	0,053	0,073	0,023	0,078	0,088	0,028	0,013	0,058	0,048	0,018	0,073	0,098
26342	0,077	0,077	0,157	0,047	0,062	0,122	0,032	0,057	0,037	0,087	0,052	0,042	0,032	0,037	0,072	0,042	0,057	0,147
26357	0,087	0,092	0,152	0,057	0,077	0,127	0,047	0,082	0,057	0,092	0,077	0,047	0,047	0,057	0,087	0,052	0,077	0,137
26493	0,100	0,080	0,185	0,065	0,075	0,205	0,055	0,060	0,040	0,090	0,100	0,050	0,040	0,050	0,085	0,055	0,070	0,215
26511	0,097	0,117	0,227	0,087	0,092	0,217	0,072	0,097	0,072	0,092	0,087	0,062	0,052	0,047	0,102	0,062	0,092	0,197
26531	0,115	0,115	0,255	0,080	0,120	0,225	0,060	0,110	0,065	0,100	0,065	0,055	0,040	0,065	0,085	0,055	0,080	0,180
26619	0,065	0,055	0,095	0,045	0,055	0,050	0,030	0,050	0,040	0,075	0,075	0,040	0,025	0,035	0,065	0,030	0,055	0,120
26846	0,075	0,090	0,145	0,055	0,070	0,140	0,030	0,100	0,090	0,100	0,045	0,045	0,025	0,035	0,070	0,045	0,130	0,170
V1234	0,145	0,120	0,280	0,105	0,165	0,250	0,075	0,130	0,080	0,130	0,150	0,110	0,070	0,075	0,160	0,105	0,145	0,285
V1200	0,125	0,105	0,300	0,090	0,100	0,270	0,055	0,105	0,090	0,110	0,105	0,090	0,050	0,060	0,130	0,085	0,100	0,210
V1100	0,120	0,110	0,290	0,100	0,100	0,285	0,065	0,135	0,090	0,145	0,075	0,085	0,050	0,040	0,110	0,075	0,135	0,255
V1000	0,125	0,080	0,235	0,065	0,080	0,250	0,040	0,075	0,070	0,085	0,060	0,085	0,035	0,040	0,110	0,055	0,075	0,235
26457	0,110	0,090	0,300	0,080	0,090	0,270	0,050	0,090	0,070	0,090	0,085	0,070	0,045	0,050	0,105	0,070	0,085	0,170
26432	0,103	0,088	0,143	0,093	0,073	0,153	0,078	0,053	0,068	0,073	0,078	0,078	0,038	0,033	0,088	0,048	0,058	0,143
26382	0,117	0,087	0,302	0,072	0,092	0,237	0,052	0,102	0,067	0,097	0,082	0,067	0,052	0,047	0,097	0,067	0,087	0,177
26283	0,112	0,077	0,152	0,067	0,072	0,157	0,042	0,057	0,042	0,057	0,082	0,072	0,027	0,037	0,092	0,037	0,067	0,177
25994	0,065	0,065	0,140	0,080	0,060	0,120	0,030	0,070	0,050	0,070	0,075	0,065	0,030	0,030	0,070	0,045	0,070	0,135

CODE	ERK1/2 (T202/Y204)	Stat1 (Y701)	Stat3 (Y705)	Akt (T308)	Akt (S473)	AMPKa (T172)	S6 Ribossomal Protein (S235/236)	mTOR (S2448)	HSP27 (S78)	Bad (S112)	p70 S6 kinase (T389)	PRAS40 (T246)	p53 (S15)	p38 (T180/Y18)	SAPK/JNK (T183/Y185)	PARP Asp214 Cleavage	Caspase-3 Asp175 Cleavage	GSK-3b (S9)
25823	0,085	0,060	0,110	0,050	0,060	0,070	0,030	0,040	0,040	0,060	0,075	0,060	0,030	0,030	0,065	0,035	0,040	0,110
25586	0,090	0,065	0,145	0,050	0,070	0,150	0,035	0,050	0,030	0,060	0,070	0,050	0,030	0,030	0,075	0,040	0,055	0,175
24882	0,105	0,080	0,125	0,055	0,080	0,095	0,040	0,060	0,040	0,080	0,095	0,065	0,030	0,040	0,090	0,050	0,065	0,150
24832	0,120	0,090	0,185	0,065	0,085	0,160	0,045	0,070	0,060	0,085	0,115	0,070	0,040	0,050	0,105	0,060	0,070	0,165
23407	0,107	0,102	0,242	0,122	0,117	0,257	0,077	0,132	0,082	0,127	0,117	0,092	0,067	0,072	0,117	0,102	0,142	0,247
23408	0,107	0,107	0,467	0,077	0,097	0,292	0,047	0,122	0,087	0,102	0,067	0,072	0,047	0,057	0,102	0,082	0,107	0,197
19108	0,125	0,080	0,175	0,065	0,085	0,165	0,040	0,070	0,065	0,085	0,125	0,085	0,040	0,050	0,115	0,065	0,080	0,170

Table 4 - Associations between patients age and the results obtained from the basic seminal analyses. [M] Moderate correlation.

Variable	Spearman correlation coefficient (p-value)
Volume	0.14 (0.4465)
pH	-0.23 (0.1928)
Concentration	-0.12 (0.5135)
No. spermatozoa	0.02 (0.9220)
Immotiled spermatozoa	0.02 (0.9134)
Non-progressive motility (type c)	0.00 (0.9914)
Slow progressive motility (type b)	0.01 (0.9760)
Fast progressive motility (type a)	0.01 (0.9722)
Total spermatozoa motility	0.00 (0.9900)
Progressive spermatozoa motility	0.01 (0.9770)
Vitality	-0.05 (0.7857)
Normal morphology	-0.03 (0.8599)
Head defects	0.04 (0.8114)
Midpiece defects	-0.43 (0.0116) [M]
Tail defects	0.08 (0.6756)
Residual cytoplasm	0.37 (0.0343) [M]
TZI	-0.23 (0.2031)
Leukocytes	-0.04 (0.8268)

Table 5 - Associations between age and the results obtained from the expression patterns of 18 well-characterized signaling molecules when phosphorylated or cleaved (PathScan® Intracellular Signaling Array) of 31 patients providing semen samples for ART or sperm analysis. [M] Moderate correlation; [S] Strong correlation.

Uniprot ID	Formerly	Presently	Recommended protein name	Phosphorylation/Cleavage	Pearson correlation coefficient	p-value
P28482	ERK1/2	MAPK1	Mitogen-activated protein kinase 1	Phospho T202/T204 Activation	-0,31348934	0,091619318
P42224	Stat1	STAT1	Signal transducer and activator of transcription 1-α/β	Phospho T701 Activation	0,25217404	0,178828774
P40763	Stat3	STAT3	Signal transducer and activator of transcription 3	Phospho T705 Activation	-0,1586831	0,402290466
P31749	Akt	AKT1	RAC-α serine/threonine-protein kinase	Phospho T308 Activation	-0,17455526	0,356234705
	AKTSer473			Phospho S473 Activation	-0,09266743	0,626227418
Q13131	AMPKα	PRKAA1	5'-AMP-activated protein kinase catalytic subunit α-1	Phospho T172	-0,17209711	0,363153603
P62753	S6 Ribosomal Protein	RPS6	40S ribosomal protein S6	Phospho S235/236 Activation	0,03248754	0,864675679
P42345	mTOR	MTOR	Serine/threonine protein kinase Mtor	Phospho S2448 Activation	0,05638103	0,767287243
P04792	HSP27	HSPB1	Heat shock protein β-1	Phospho S78 Activation	-0,08591658	0,6516833
Q92934	Bad	BAD	Bcl2-associated agonist of cell death	Phospho S112 Activation	0,10126071	0,594426416
P23443	p70 S6 kinase	RPS6KB1	Ribossomal protein S6 kinase β-1	Phospho T389 Activation	-0,36520541 [M]	0,047204113
Q96B36	PRAS40	AKT1S1	Proline-rich AKT1 substrate 1	Phospho T246 Activation	-0,5383929 [S]	0,002146602
P04637	p53	TP53	Cellular tumor antigen p53	Phospho S15 Activation	-0,02172066	0,909295688

Uniprot ID	Formerly	Presently	Recommended protein name	Phosphorylation/Cleavage	Pearson correlation coefficient	p-value
Q16539; Q15759; P53778; O15264	p38	MAPK14/11/12/13	Mirogen-activated protein kinase 14/11/12/13	Phospho T180/Y182 Activation	0,10271796	0,589103587
	SAPK/JNK				-0,29666033	0,111405889
P09874	PARP	PARP1	Poly [ADP-ribose] polymerase 1	Cleavage D214 Inhibition	-0,284881	0,127041486
P42574	Caspase-3	CASP3	Caspase-3	Cleavage D175 Inhibition	-0,0092279	0,961400198
P49841	GSK-3β	GSK3B	Glycogen synthase kinase-3 β	Phospho S9 Inhibition	-0,09185677	0,629262825

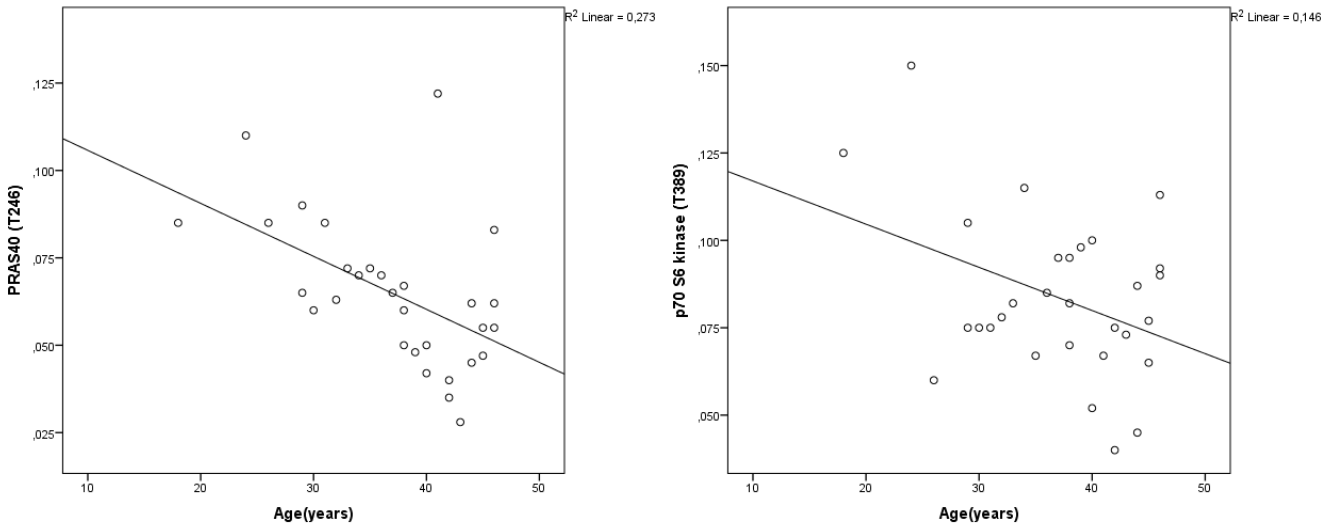

Figure 7 - Scatter plots and the regression lines: Correlation between age and the signaling proteins PRAS40 and p70 S6 kinase.

4. Discussion

Increasing evidence indicates that advanced paternal age is associated with dysfunction in semen traits, but the molecular mechanisms responsible for age-dependent decline in spermatozoa quality and reproductive outcomes are far from being identified (Sharma et al., 2015; Kidd, Eskenazi and Wyrobek, 2001; Eskenazi et al., 2003). The main objective of this study was to evaluate the impact of aging on human spermatozoa signaling proteins.

We started by analysing the correlation between age and basic seminal parameters. We showed a negative correlation between age and the percentage of midpiece defects. This result was not consistent with the literature, since a mitochondrial respiratory inefficiency associated with increased age was expected, increasing the probability of structural defects in spermatozoa (Amaral and Ramalho-Santos, 2009; Uzhachenko et al., 2017; Darr et al., 2017). A study concerning the role of mitochondria on spermatozoa function, revealed a strong negative correlation between mitochondrial respiration and defects in the midpiece where mitochondria are exclusive, proposing that structural anomalies were accompanied by a decrease in mitochondrial respiration (Ferramosca and Zara, 2017). A test to evaluate the efficiency of mitochondrial respiration could be done to confirm our results.

Also, the present study showed a significant positive correlation between age and the presence of excess residual cytoplasm (ERC) in spermatozoa, as previously described in the literature (Gomez, et al., 1996; Rengan, et al., 2012). ERC is defined as a retention of cytoplasm around the midpiece during spermiogenesis (Cooper et al., 2004; Rengan et al., 2012). Some studies showed an association between the ERC and varicocele (Zini et al., 2000; Zini et al., 1999). In the present study, it iwas not possible to establish such correlation since only one patient presented varicocele. Mikolcevic et al., 2012, performed a study with mice and found a positive correlation between cyclin-dependent kinase 16 (CDK16) deficiency and spermatozoa with ERC. Also, they showed that CDK16 is expressed in the testicles and required for normal spermatogenesis, suggesting a role of CDK16 in spermiation.

The excess of cytoplasm enzymes, like glucose-6-phosphate dehydrogenase (G6PDH) results in a high production of nicotinamide adenine dinucleotide phosphate (NADPH), contributing to the production of H_2O_2 responsible for the peroxidative damage in spermatozoa (Rengan et al., 2012), affecting morphology, namely the midpiece. Therefore, this study presents results accordingly to the consulted literature.

Other basic seminal parameters (volume, pH, motility, concentration, vitality and presence of leukocytes) did not show a significant correlation with age in our study, probably due to the relatively small overall sample size, which limits the power of the study. However, the literature indicates that are an inverse relationship between semen quality/parameters and male age (Levitas et al., 2007; Plastira et al., 2007; Eskenazi et al., 2003; Stone et al., 2013; Centola and Eberly, 1999). Selection bias is also to be considered since all volunteers were patients from a fertility clinic.

Posttranslational modifications and signal cascades are essential for spermatozoa function. Protein phosphorylation is a fundamental mechanism for sperm motility, capacitation and acrosome reaction (Visconti and Kopf, 1998). The present study examined 18 well-characterized signaling proteins for their phosphorylation or cleavage status and then they activity levels were correlated with age.

Proline-rich AKT1 substrate 1 (PRAS 40) is a protein rich in proline (has 15% proline residues), substrate of the mammalian target of rapamycin complex 1 (mTORC1) and acts at the intersection of the protein kinase B (Akt) and mTOR signal pathways (Chong, 2016; Wiza, Nascimento and Ouwens, 2012; Oshiro et al., 2007; Nascimento et al., 2010). The mTOR and its regulatory proteins constitute two complexes, mTORC1 and mTORC2. mTORC1 is composed of several subunits: mTOR, PRAS40, regulatory associated protein of mTOR (Raptor) necessary to create this complex, mammalian lethal with sec-13 protein 8 (mLST8), DEP domain-containing mTOR-interacting protein (Deptor), DNA damage-binding protein 1-cullin 4 (CUL4), telomere length regulation protein 2 (Tel2) and Tel2-interacting protein 1 (Tti1) (Zhong-Chong, 2015). Phosphorylation of PRAS40 by PKB leads to its detachment activating the mTORC1 complex (Wang, Harris and Lawrence, 2008). Recent studies showed that the activation of mTORC1 complex promotes the differentiation of spermatogonial stem cells

(maintenance and proliferation) (Xiong et al., 2015; Xu et al., 2016). Busada et al., 2015, demonstrated that retinoic acid (a metabolite of vitamin A that induces Sertoli cell paracrine signals for spermatogonial differentiation (Raverdeau et al., 2012), increases phosphorylation of mTORC1. This study indicates that retinoic acid is essential to cell proliferation. Wang et al., 2016, proved that mTORC1 activation promotes spermatogonial differentiation and that if not inhibited, it promotes the early depletion of germ cells, compromising spermatogenesis and consequently inducing infertility. Xu et al., 2016, performed a study with mice (Sprague Dawley rats) that showed that mTORC1 inhibition causes the atrophy of seminiferous tubules, resulting in reduced sperm production, demonstrating the role of mTORC1 in spermatogenesis. PRAS40 is involved in multiple cell processes, like apoptosis and oxidative stress (Chong, 2016). During oxidative stress, mTOR signaling pathways can become depressed and lead to cell injury (Faghiri and Bazan, 2010). PRAS40 disfunctions are also associated with several diseases as diabetes mellitus, cardiovascular diseases, cancer and neurological diseases (Chong, 2016).

Ribosomal protein S6 kinase beta-1 (P70 S6) kinase is a mitogen activated protein-ser/thr protein kinase involved in actin cytoskeleton dynamics in human spermatozoa and required to regulate spermatogenesis (Ip and Wong, 2012). Chiang and Abraham, 2005, showed that P70 S6 kinase can be responsible for mTOR activation by phosphorylation in S2448. mTOR plays an important role in spermatogenesis by regulating p70 S6 kinase activation (Xu et al., 2016). Silva et al., 2015, performed a study that analysed signaling proteins in human spermatozoa as indicators for sperm quality evaluation. They described that in human spermatozoa the levels of active P70 S6 kinase were positively correlated with the percentage of normal spermatozoa and negatively correlated with the percentage of sperm DNA fragmentation. Flegel et al., 2016, demonstrated that stimulation with the G protein-coupled receptor 18 (GPR18) ligand induced a phosphorylation of P70 S6 kinase. This phosphorylation possibly was responsible for play actin polymerization in human sperm, as well the induction of the acrosome reaction. Also, in a recent study, performed with male Sprague Dawley rats showed that immunosuppressants like rapamycin inhibit spermatogenesis if phosphorylation of P70 S6 kinase was suppressed, reducing the sperm cell number (Liu

et al., 2017). The present study shows a negative correlation between the levels of activated P70 S6 kinase and age, demonstrating that in men with increased age the levels of activated P70 S6 kinase were reduced, which may negatively impact key processes such as spermatogenesis and DNA integrity.

To summarize, in the present study a negative correlation between the levels of activated P70 S6 kinase and PRAS40 with age becomes evident, demonstrating that probably Akt/mTOR/P70 S6 kinase signal pathway (figure 8) is compromised with age: PRAS40 is not phosphorylated, inhibiting the activation of the mTORC1 complex, not allowing P70 S6 kinase phosphorylation. Consequently, the ribossomal protein S6 (RPS6) kinase phosphorylation decreases, stopping spermatogonial proliferation and differentiation, which may negatively impact key processes such as spermatogenesis and DNA integrity (Chong, 2016; Xu et al., 2016). Akt/PRAS40/mTORC1 signaling pathway has an important role in cell bioactivities and might aid in the development of novel therapeutic strategies for male reproductive infertility problems.

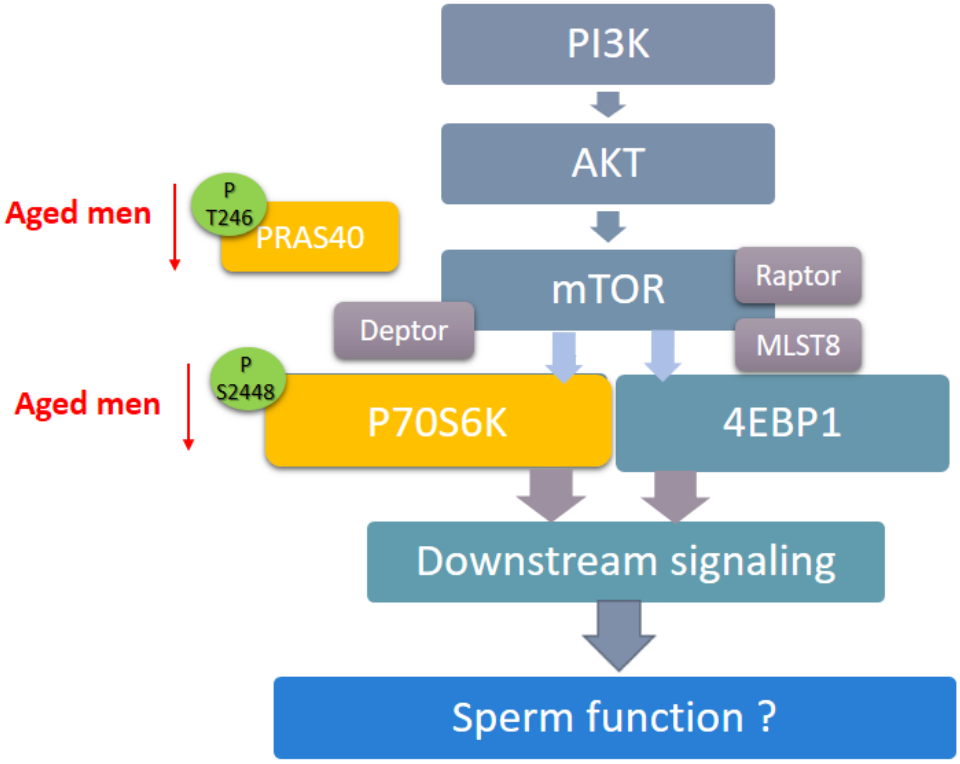

Figure 8 - Schematic representation of PI3K/AKT1/mTORC1/P70-S6K/RPS6KB1 and its described role on spermatogonia proliferation. PI3K phosphorylates PDK1 which in turn, phosphorylates AKT. AKT stimulates the activation of the mTORC1 complex allowing P70-S6K phosphorylation. RPS6B1 phosphorylation leads to spermatogonial proliferation and differentiation. PI3K: phosphatidylinositide 3-kinase; AKT: protein kinase B; mTOR: mammalian target of rapamycin complex; P70-S6KB1: ribosomal protein S6 kinase β1; 4EBP1: eukaryotic translation initiation factor 4E binding protein 1; RPS6KB1: ribossomal protein S6 β1.

5. References

- Abou-haila, A., & Tulsiani, D. R. (2009). Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. Archives of biochemistry and biophysics, 485(1), 72-81
- Agarwal, A., & Said, T. M. (2003). Role of sperm chromatin abnormalities and DNA damage in male infertility. Human reproduction update, 9(4), 331-345.
- Aitken, R. J. (2017). Reactive oxygen species as mediators of sperm capacitation and pathological damage. Molecular Reproduction and Development.
- Alasmari, W., Costello, S., Correia, J., Oxenham, S. K., Morris, J., Fernandes, L., & Barratt, C. L. (2013). Ca2+ signals generated by CatSper and Ca2+ stores regulate different behaviors in human sperm. Journal of Biological Chemistry, 288(9), 6248-6258.
- Amaral, S., & Ramalho-Santos, J. (2009). Aging, mitochondria and male reproductive function. Current aging science, 2(3), 165-173.
- Bailey, J. L. (2010). Factors regulating sperm capacitation. Systems biology in reproductive medicine, 56(5), 334-348.
- Balhorn, R. (2007). The protamine family of sperm nuclear proteins. Genome biology, 8(9), 227.
- Bearer, E. L., & Friend, D. S. (1990). Morphology of mammalian sperm membranes during differentiation, maturation, and capacitation. Microscopy Research and Technique, 16(4), 281-297.
- Bellastella, G., Cooper, T. G., Battaglia, M., Ströse, A., Torres, I., Hellenkemper, B., & Sinisi, A. A. (2010). Dimensions of human ejaculated spermatozoa in Papanicolaou-stained seminal and swim-up smears obtained from the Integrated Semen Analysis System (ISAS®). Asian journal of andrology, 12(6), 871.
- Belloc, S., Hazout, A., Zini, A., Merviel, P., Cabry, R., Chahine, H., & Benkhalifa, M. (2014). How to overcome male infertility after 40: Influence of paternal age on fertility. Maturitas, 78(1), 22-29.
- Benton, L., Shan, L. X., & Hardy, M. P. (1995). Differentiation of adult Leydig cells. The Journal of steroid biochemistry and molecular biology, 53(1), 61-68.
- Béziau, D. M., Toussaint, F., Blanchette, A., Dayeh, N. R., Charbel, C., Tardif, J. C., & Ledoux, J. (2015). Expression of phosphoinositide-specific phospholipase C isoforms in native endothelial cells. PloS one, 10(4), e0123769.
- Brahem, S., Mehdi, M., Landolsi, H., Mougou, S., Elghezal, H., & Saad, A. (2011). Semen parameters and sperm DNA fragmentation as causes of recurrent pregnancy loss. Urology, 78(4), 792-796.
- Bras, M., Lens, J. W., Piederiet, M. H., Rijnders, P. M., Verveld, M., & Zeilmaker, G. H. (1996). Ivf lab: Laboratory aspects of in-vitro fertilization. NV Organon, Oss.
- Breitbart, H. (2002). Intracellular calcium regulation in sperm capacitation and acrosomal reaction. Molecular and cellular endocrinology, 187(1), 139-144.
- Breitbart, H. (2003). Signaling pathways in sperm capacitation and acrosome reaction. Cellular and molecular biology. Paris-Wegmann, 49(3), 321-328.

- Brewer, L., Corzett, M., & Balhorn, R. (2002). Condensation of DNA by spermatid basic nuclear proteins. Journal of Biological Chemistry, 277(41), 38895-38900.
- Buffone, M. G., Wertheimer, E. V., Visconti, P. E., & Krapf, D. (2014). Central role of soluble adenylyl cyclase and cAMP in sperm physiology. Biochimica Et Biophysica Acta (BBA)-Molecular Basis of Disease, 1842(12), 2610-2620.
- Burton, K. A., & McKnight, G. S. (2007). PKA, germ cells, and fertility. Physiology, 22(1), 40-46.
- Busada, J. T., Chappell, V. A., Niedenberger, B. A., Kaye, E. P., Keiper, B. D., Hogarth, C. A., & Geyer, C. B. (2015). Retinoic acid regulates Kit translation during spermatogonial differentiation in the mouse. Developmental biology, 397(1), 140-149.
- Calvel, P., Rolland, A. D., Jégou, B., & Pineau, C. (2010). Testicular postgenomics: targeting the regulation of spermatogenesis. Philosophical Transactions of the Royal Society of London B: Biological Sciences, 365(1546), 1481-1500.
- Centola, G. M., & Eberly, S. (1999). Seasonal variations and age-related changes in human sperm count, motility, motion parameters, morphology, and white blood cell concentration. Fertility and sterility, 72(5), 803-808.
- Chakrabarti, R., Cheng, L., Puri, P., Soler, D., & Vijayaraghavan, S. (2007). Protein phosphatase PP1γ2 in sperm morphogenesis and epididymal initiation of sperm motility. Asian journal of andrology, 9(4), 445-452.
- Chiang, G. G., & Abraham, R. T. (2005). Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase. Journal of Biological Chemistry, 280(27), 25485-25490.
- Chong, Z. Z. (2016). Targeting PRAS40 for multiple diseases. *Drug discovery today*, *21*(8), 1222-1231.
- Chung, J. J., Miki, K., Kim, D., Shim, S. H., Shi, H. F., Hwang, J. Y., & Clapham, D. E. (2017). CatSperζ regulates the structural continuity of sperm Ca2+ signaling domains and is required for normal fertility. eLife, 6, e23082.
- Clermont, Y., & Huckins, C. (1961). Microscopic anatomy of the sex cords and seminiferous tubules in growing and adult male albino rats. Developmental Dynamics, 108(1), 79-97.
- Cocuzza, M., Athayde, K. S., Agarwal, A., Sharma, R., Pagani, R., Lucon, A. M., & Hallak, J. (2008). Age-related increase of reactive oxygen species in neat semen in healthy fertile men. Urology, 71(3), 490-494.
- Cooke, H. J., & Saunders, P. T. (2002). Mouse models of male infertility. Nature reviews. Genetics, 3(10), 790.
- Cooper, T. G. (2011). The epididymis, cytoplasmic droplets and male fertility. Asian journal of andrology, 13(1), 130.
- Cooper, T. G. (2012). The epididymis, sperm maturation and fertilisation. Springer Science & Business Media.
- Cooper, T. G., Yeung, C. H., Fetic, S., Sobhani, A., & Nieschlag, E. (2004). Cytoplasmic droplets are normal structures of human sperm but are not well preserved by routine procedures for assessing sperm morphology. Human reproduction, 19(10), 2283-2288.
- Cruz, D. F., Freitas, M. J., Patrício, A., & Fardilha, M. (2014). Vias de sinalização reguladoras das funções do espermatozoide. Revista Internacional de Andrología, 12(3), 104-111.

- Damassa, D. A., Kobashigawa, D., Smith, E. R., & Davidson, J. M. (1976). Negative Feedback Control of LH by Testosterone: A Quantitative Study in Male Rats1. Endocrinology, 99(3), 736-742.
- Darr, C. R., Moraes, L. E., Scanlan, T. N., Baumber-Skaife, J., Loomis, P. R., Cortopassi, G. A., & Meyers, S. A. (2017). Sperm Mitochondrial Function is Affected by Stallion Age and Predicts Post-Thaw Motility. Journal of Equine Veterinary Science, 50, 52-61.
- De Lamirande, E., & Gagnon, C. (1995). Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. Human Reproduction, 10(suppl_1), 15-21.
- De Lamirande, E., Leclerc, P., & Gagnon, C. (1997). Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Molecular human reproduction, 3(3), 175-194.
- Desai, N., Ludgin, J., Sharma, R., Anirudh, R. K., & Agarwal, A. (2017). Female and male gametogenesis. In *Clinical Reproductive Medicine and Surgery* (pp. 19-45). Springer, Cham.
- Ellerman, D. A., Pei, J., Gupta, S., Snell, W. J., Myles, D., & Primakoff, P. (2009). Izumo is part of a multiprotein family whose members form large complexes on mammalian sperm. Molecular reproduction and development, 76(12), 1188-1199.
- Eskenazi, B., Wyrobek, A. J., Sloter, E., Kidd, S. A., Moore, L., Young, S., & Moore, D. (2003). The association of age and semen quality in healthy men. Human Reproduction, 18(2), 447-454.
- Evenson, D. P., Brannian, J., Hansen, K., Kasperson, K. M., & Christianson, J. (2014). Relationships between sperm DNA fragmentation, age of donors and patients and children with psychic disorders. Fertility and Sterility, 102(3), e97.
- Faghiri, Z., & Bazan, N. G. (2010). PI3K/Akt and mTOR/p70S6K pathways mediate neuroprotectin D1-induced retinal pigment epithelial cell survival during oxidative stress-induced apoptosis. *Experimental eye research*, *90*(6), 718-725.
- Fardilha M., Ferreira M., Pelech S., Vieira S., Rebelo S., Korrodi-Gregorio L., Sousa M., Barros A., Silva V., da Cruz e Silva O.A. & da Cruz e Silva E.F. (2013) "Omics" of human sperm: profiling protein phosphatases. OMICS 17, 460–472.
- Fardilha, M., Esteves, S. L., Korrodi-Gregório, L., Pelech, S., da Cruz e Silva, O. A., & da Cruz e Silva, E. (2011). Protein phosphatase 1 complexes modulate sperm motility and present novel targets for male infertility. MHR: Basic science of reproductive medicine, 17(8), 466-477.
- Fardilha, M., V. Silva, J., & Conde, M. (2015). Reprodução humana masculina: príncipios fundamentais. ARC Publishing.
- Ferramosca, A., & Zara, V. (2017). Mitochondria and fertility: the mitochondria critical role on spermatozoa function. JDREAM. Journal of interDisciplinary REsearch Applied to Medicine, 1(1), 21-26.
- FFMS: Fundação Francisco Manuel dos Santos. (2016). Nascer em Portugal (updated 2017 December 1. Available from: http://nasceremportugal.ffms.pt/#ter-ou-nao-ter
- Flegel, C., Vogel, F., Hofreuter, A., Wojcik, S., Schoeder, C., Kieć-Kononowicz, K., & Hatt, H. (2016). Characterization of non-olfactory GPCRs in human sperm with a focus on GPR18. Scientific reports, 6, 32255.

- Fukami, K., Inanobe, S., Kanemaru, K., & Nakamura, Y. (2010). Phospholipase C is a key enzyme regulating intracellular calcium and modulating the phosphoinositide balance. Progress in lipid research, 49(4), 429-437.
- Gomez, E., Buckingham, D. W., Brindle, J., Lanzafame, F., Irvine, D. S., & Aitken, R. J. (1996). Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. Journal of andrology, 17(3), 276-287.
- Griswold, M. D. (1998). The central role of Sertoli cells in spermatogenesis. In Seminars in cell & developmental biology (Vol. 9, No. 4, pp. 411-416). Academic Press.
- Hammoud, A. O., Wilde, N., Gibson, M., Parks, A., Carrell, D. T., & Meikle, A. W. (2008). Male obesity and alteration in sperm parameters. Fertility and sterility, 90(6), 2222-2225.
- Harris, I. D., Fronczak, C., Roth, L., & Meacham, R. B. (2011). Fertility and the aging male. Reviews in urology, 13(4), e184.
- Haschek, W. M., Rousseaux, C. G., & Wallig, M. A. (Eds.). (2009). Fundamentals of toxicologic pathology. Academic Press.
- Heller, C. G., & Clermont, Y. (1963). Spermatogenesis in man: an estimate of its duration. Science, 140(3563), 184-186.
- Huang, V. W., Lee, C. L., Lee, Y. L., Lam, K. K., Ko, J. K., Yeung, W. S. & Chiu, P. C. (2015). Sperm fucosyltransferase-5 mediates spermatozoa–oviductal epithelial cell interaction to protect human spermatozoa from oxidative damage. MHR: Basic science of reproductive medicine, 21(6), 516-526.
- Huang, V. W., Zhao, W., Lee, C. L., Lee, C. Y., Lam, K. K., Ko, J. K. & Chiu, P. C. (2013). Cell membrane proteins from oviductal epithelial cell line protect human spermatozoa from oxidative damage. Fertility and sterility, 99(5), 1444-1452.
- Huang, Z., & Vijayaraghavan, S. (2004). Increased phosphorylation of a distinct subcellular pool of protein phosphatase, PP1γ2, during epididymal sperm maturation. Biology of reproduction, 70(2), 439-447.
- Ickowicz, D., Finkelstein, M., & Breitbart, H. (2012). Mechanism of sperm capacitation and the acrosome reaction: role of protein kinases. Asian journal of andrology, 14(6), 816.
- Inaba, K. (2003). Molecular architecture of the sperm flagella: molecules for motility and signaling. Zoological science, 20(9), 1043-1056.
- INE: Instituto Nacional de Estatistica. (2014). Quatro décadas de declínio de fecundidade em Portugal. Inquérito à Fecundidade 2013.
- Ip, C. K., & Wong, A. S. (2012). p70 S6 kinase and actin dynamics: a perspective. Spermatogenesis, 2(1), 44-52.
- Itach, S. B. S., Finklestein, M., Etkovitz, N., & Breitbart, H. (2012). Hyper-activated motility in sperm capacitation is mediated by phospholipase D-dependent actin polymerization. Developmental biology, 362(2), 154-161.
- Ivell, R. (2007). Lifestyle impact and the biology of the human scrotum. Reproductive biology and endocrinology, 5(1), 15.
- Jefferys, A., Siassakos, D., & Wardle, P. (2012). The management of retrograde ejaculation: a systematic review and update. Fertility and sterility, 97(2), 306-312.
- Jin, L., Wang, W., & Fang, G. (2014). Targeting protein-protein interaction by small molecules. Annual review of pharmacology and toxicology, 54, 435-456.

- Jungwirth, A., Giwercman, A., Tournaye, H., Diemer, T., Kopa, Z., Dohle, G., & EAU Working Group on Male Infertility. (2012). European Association of Urology guidelines on Male Infertility: the 2012 update. European urology, 62(2), 324-332.
- Kanakis, G. A., & Goulis, D. D. (2015). Male contraception: a clinically-oriented review. Hormones (Athens), 14(4), 598.
- Kassebaum, N. J., Bertozzi-Villa, A., Coggeshall, M. S., Shackelford, K. A., Steiner, C., Heuton, K. R., & Templin, T. (2014). Global, regional, and national levels and causes of maternal mortality during 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. The Lancet, 384(9947), 980-1004.
- Kaupp, U. B., & Strünker, T. (2017). Signaling in sperm: more different than similar. Trends in cell biology, 27(2), 101-109.
- Keel, B. A. (2006). Within-and between-subject variation in semen parameters in infertile men and normal semen donors. Fertility and sterility, 85(1), 128-134.
- Kerr, J. B. (1991). Ultrastructure of the seminiferous epithelium and intertubular tissue of the human testis. Microscopy Research and Technique, 19(2), 215-240.
- Kerr, J. B. (1992). Functional cytology of the human testis. Baillière's clinical endocrinology and metabolism, 6(2), 235-250.
- Kidd, S. A., Eskenazi, B., & Wyrobek, A. J. (2001). Effects of male age on semen quality and fertility: a review of the literature. Fertility and sterility, 75(2), 237-248.
- Kimura, M., Kim, E., Kang, W., Yamashita, M., Saigo, M., Yamazaki, T., & Baba, T. (2009). Functional roles of mouse sperm hyaluronidases, HYAL5 and SPAM1, in fertilization. Biology of reproduction, 81(5), 939-947.
- Koh, S. A., Sanders, K., & Burton, P. (2016). Effect of male age on oxidative stress markers in human semen. Journal of Reproductive Biotechnology and Fertility, 5, 2058915816673242.
- Korrodi-Gregório, L., Ferreira, M., Vintém, A. P., Wu, W., Muller, T., Marcus, K., & e Silva, E. F. D. C. (2013). Identification and characterization of two distinct PPP1R2 isoforms in human spermatozoa. BMC cell biology, 14(1), 15.
- Leahy, T., & Gadella, B. M. (2015). New insights into the regulation of cholesterol efflux from the sperm membrane. Asian journal of andrology, 17(4), 561.
- Lee, D., Moawad, A. R., Morielli, T., Fernandez, M. C., & O'flaherty, C. (2017). Peroxiredoxins prevent oxidative stress during human sperm capacitation. MHR: Basic science of reproductive medicine, 23(2), 106-115.
- Leemans, B., Gadella, B. M., Stout, T. A., Sostaric, E., De Schauwer, C., Nelis, H., & Van Soom, A. (2016). Combined albumin and bicarbonate induces head-to-head sperm agglutination which physically prevents equine sperm–oviduct binding. Reproduction, 151(4), 313-330.
- Levitas, E., Lunenfeld, E., Weisz, N., Friger, M., & Potashnik, G. (2007). Relationship between age and semen parameters in men with normal sperm concentration: analysis of 6022 semen samples. Andrologia, 39(2), 45-50.
- Lindemann, C. B., & Lesich, K. A. (2010). Flagellar and ciliary beating: the proven and the possible. J Cell Sci, 123(4), 519-528.
- Lishko, P. V., Botchkina, I. L., & Kirichok, Y. (2011). Progesterone activates the principal Ca2+ channel of human sperm. Nature, 471(7338), 387-391.

- Liu, S., Huang, L., Geng, Y., He, J., Chen, X., Xu, H., & Liu, X. (2017). Rapamycin inhibits spermatogenesis by changing the autophagy status through suppressing mechanistic target of rapamycin-p70S6 kinase in male rats. Molecular medicine reports, 16(4), 4029-4037.
- López-González, I., Torres-Rodríguez, P., Sánchez-Carranza, O., Solís-López, A., Santi, C. M., Darszon, A., & Treviño, C. L. (2014). Membrane hyperpolarization during human sperm capacitation. Molecular human reproduction, 20(7), 619-629.
- Marchiani, S., Tamburrino, L., Muratori, M., & Baldi, E. (2017). Epididymal Sperm Transport and Fertilization. Endocrinology of the Testis and Male Reproduction, 1-22.
- Martan, J. (1969). Epididymal histochemistry and physiology. Biology of reproduction, 134-154.
- Martínez, P., & Morros, A. (1996). Membrane lipid dynamics during human sperm capacitation. Front Biosci, 1, d103-17.
- Mascarenhas, M. N., Flaxman, S. R., Boerma, T., Vanderpoel, S., & Stevens, G. A. (2012). National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. PLoS medicine, 9(12), e1001356.
- Maya, W., Berdugo, J., & Jaramillo, Á. (2009). The effects of male age on semen parameters: analysis of 1364 men attending an andrology center. The Aging Male, 12(4), 100-103.
- McCudden, C. R., Hains, M. D., Kimple, R. J., Siderovski, D. P., & Willard, F. S. (2005). G-protein signaling: back to the future. Cellular and molecular life sciences, 62(5), 551-577.
- McLachlan, R. I., Rajpert-De Meyts, E., Hoei-Hansen, C. E., De Kretser, D. M., & Skakkebaek, N. E. (2006). Histological evaluation of the human testis—approaches to optimizing the clinical value of the assessment: mini review. Human Reproduction, 22(1), 2-16.
- Middendorff, R., Müller, D., Mewe, M., Mukhopadhyay, A. K., Holstein, A. F., & Davidoff, M. S. (2002). The tunica albuginea of the human testis is characterized by complex contraction and relaxation activities regulated by cyclic GMP. The Journal of Clinical Endocrinology & Metabolism, 87(7), 3486-3499.
- Miki, K., Willis, W. D., Brown, P. R., Goulding, E. H., Fulcher, K. D., & Eddy, E. M. (2002). Targeted disruption of the Akap4 gene causes defects in sperm flagellum and motility. Developmental biology, 248(2), 331-342.
- Mikolcevic, P., Sigl, R., Rauch, V., Hess, M. W., Pfaller, K., Barisic, M., & Geley, S. (2012). Cyclin-dependent kinase 16/PCTAIRE kinase 1 is activated by cyclin Y and is essential for spermatogenesis. Molecular and cellular biology, 32(4), 868-879.
- Miranda PV, Allaire A, Sosnik J & Visconti PE. (2009) Localization of low-density detergent-resistant membrane proteins in intact and acrosome-reacted mouse sperm. Biol Reprod 80, 897–904.
- Molina, R. I., Martini, A. C., Tissera, A., Olmedo, J., Senestrari, D., de Cuneo, M. F., & Ruiz, R. D. (2010). Semen quality and aging: analysis of 9.168 samples in Cordoba. Argentina. Arch Esp Urol, 63(3), 214-22.
- Moskovtsev, S. I., Willis, J., & Mullen, J. B. M. (2006). Age-related decline in sperm deoxyribonucleic acid integrity in patients evaluated for male infertility. Fertility and sterility, 85(2), 496-499.

- Mukhopadhyay, D., Varghese, A. C., Pal, M., Banerjee, S. K., Bhattacharyya, A. K., Sharma, R. K., & Agarwal, A. (2010). Semen quality and age-specific changes: a study between two decades on 3,729 male partners of couples with normal sperm count and attending an andrology laboratory for infertility-related problems in an Indian city. Fertility and sterility, 93(7), 2247-2254.
- Nakamura, Y., & Fukami, K. (2017). Regulation and physiological functions of mammalian phospholipase C. The Journal of Biochemistry, 161(4), 315-321.
- Nascimento, E. B., Snel, M., Guigas, B., van der Zon, G. C., Kriek, J., Maassen, J. A., & Ouwens, D. M. (2010). Phosphorylation of PRAS40 on Thr246 by PKB/AKT facilitates efficient phosphorylation of Ser183 by mTORC1. Cellular Signaling, 22(6), 961-967.
- Nishibe, S., Wahl, M. I., Hernandez-Sotomayor, S. T., Tonks, N. K., Rhee, S. G., & Carpenter, G. (1990). Increase of the catalytic activity of phospholipase C-gamma 1 by tyrosine phosphorylation. Science, 250(4985), 1253-1257.
- Nistal, M., Paniagua, R., Regadera, J., Santamaria, L., & Amat, P. (1986). A quantitative morphological study of human Leydig cells from birth to adulthood. Cell and tissue research, 246(2), 229-236.
- O'Flaherty, C., de Lamirande, E., & Gagnon, C. (2005). Reactive oxygen species and protein kinases modulate the level of phospho-MEK-like proteins during human sperm capacitation. Biology of reproduction, 73(1), 94-105.
- O'flaherty, C., de Lamirande, E., & Gagnon, C. (2006). Reactive oxygen species modulate independent protein phosphorylation pathways during human sperm capacitation. Free Radical Biology and Medicine, 40(6), 1045-1055.
- Oliva, R. (2006). Protamines and male infertility. Human reproduction update, 12(4), 417-435.
- Olson, G. E., & Sammons, D. W. (1980). Structural chemistry of outer dense fibers of rat sperm. Biology of reproduction, 22(2), 319-332.
- Oshiro, N., Takahashi, R., Yoshino, K. I., Tanimura, K., Nakashima, A., Eguchi, S., & Kikkawa, U. (2007). The proline-rich Akt substrate of 40 kDa (PRAS40) is a physiological substrate of mammalian target of rapamycin complex 1. Journal of Biological Chemistry, 282(28), 20329-20339.
- Pelletier, R., & Byers, S. W. (1992). The blood-testis barrier and Sertoli cell junctions: Structural considerations. Microscopy research and technique, 20(1), 3-33.
- Plastira, K., Msaouel, P., Angelopoulou, R., Zanioti, K., Plastiras, A., Pothos, A., & Mantas, D. (2007). The effects of age on DNA fragmentation, chromatin packaging and conventional semen parameters in spermatozoa of oligoasthenoteratozoospermic patients. Journal of assisted reproduction and genetics, 24(10), 437-443.
- Pordata. (2016). Updated 2017 8 December. Available from: https://www.pordata.pt/Portugal/Idade+m%C3%A9dia+da+m%C3%A3e+ao+nascimento+do+primeiro+filho-805.
- Prince, F. P. (2001). The triphasic nature of Leydig cell development in humans, and comments on nomenclature. Journal of Endocrinology, 168(2), 213-216.

- Qi, H., Moran, M. M., Navarro, B., Chong, J. A., Krapivinsky, G., Krapivinsky, L., & Clapham, D. E. (2007). All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. Proceedings of the National Academy of Sciences, 104(4), 1219-1223.
- Rato, L., Alves, M. G., Socorro, S., Duarte, A. I., Cavaco, J. E., & Oliveira, P. F. (2012). Metabolic regulation is important for spermatogenesis. Nature Reviews Urology, 9(6), 330-338.
- Raverdeau, M., Gely-Pernot, A., Féret, B., Dennefeld, C., Benoit, G., Davidson, I., & Ghyselinck, N. B. (2012). Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. Proceedings of the National Academy of Sciences, 109(41), 16582-16587.
- Rengan, A. K., Agarwal, A., van der Linde, M., & du Plessis, S. S. (2012). An investigation of excess residual cytoplasm in human spermatozoa and its distinction from the cytoplasmic droplet. Reproductive Biology and Endocrinology, 10(1), 92.
- Sadler, T. W. (2011). Langman's medical embryology. Lippincott Williams & Wilkins Inc. Editions
- Saez, F., Frenette, G., & Sullivan, R. (2003). Epididymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells. Journal of andrology, 24(2), 149-154.
- Schwartz, N., Verma, A., Bivens, C. B., Schwartz, Z., & Boyan, B. D. (2016). Rapid steroid hormone actions via membrane receptors. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1863(9), 2289-2298.
- Sharma, R., Agarwal, A., Rohra, V. K., Assidi, M., Abu-Elmagd, M., & Turki, R. F. (2015). Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. Reproductive Biology and Endocrinology, 13(1), 35.
- Silva, J. V., Freitas, M. J., Correia, B. R., Korrodi-Gregório, L., Patrício, A., Pelech, S., & Fardilha, M. (2015). Profiling signaling proteins in human spermatozoa: biomarker identification for sperm quality evaluation. Fertility and sterility, 104(4), 845-856.
- Silva, J. V., Freitas, M. J., & Fardilha, M. (2014). Phosphoprotein phosphatase 1 complexes in spermatogenesis. Current molecular pharmacology, 7(2), 136-146.
- Silva, L.F., & Carvalho, L.F. (2010). Atualizações na infertilidade masculina. Sociedade Portuguesa de Andrologia, 7-18.
- Sloter, E., Schmid, T. E., Marchetti, F., Eskenazi, B., Nath, J., & Wyrobek, A. J. (2006). Quantitative effects of male age on sperm motion. Human Reproduction, 21(11), 2868-2875.
- Somanath, P. R., Jack, S. L., & Vijayaraghavan, S. (2004). Changes in Sperm Glycogen Synthase Kinase-3 Serine Phosphorylation and Activity Accompany Motility Initiation and Stimulation. Journal of andrology, 25(4), 605-617.
- Stone, B. A., Alex, A., Werlin, L. B., & Marrs, R. P. (2013). Age thresholds for changes in semen parameters in men. Fertility and sterility, 100(4), 952-958.
- Strünker, T., Goodwin, N., Brenker, C., Kashikar, N. D., Weyand, I., Seifert, R., & Kaupp, U. B. (2011). The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. Nature, 471(7338), 382-386.
- Sullivan, R., Frenette, G., & Girouard, J. (2007). Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. Asian journal of andrology, 9(4), 483-491.

- Tash, J. S., & Bracho, G. E. (1994). Regulation of sperm motility: emerging evidence for a major role for protein phosphatases. Journal of andrology, 15(6), 505-509.
- Terada, Y., Luetjens, C. M., Sutovsky, P., & Schatten, G. (2000). Atypical decondensation of the sperm nucleus, delayed replication of the male genome, and sex chromosome positioning following intracytoplasmic human sperm injection (ICSI) into golden hamster eggs: does ICSI itself introduce chromosomal anomalies?. Fertility and sterility, 74(3), 454-460.
- Thundathil, J., De Lamirande, E., & Gagnon, C. (2002). Different signal transduction pathways are involved during human sperm capacitation induced by biological and pharmacological agents. Molecular human reproduction, 8(9), 811-816.
- Tsukita, S., Furuse, M., & Itoh, M. (2001). Multifunctional strands in tight junctions. Nature reviews Molecular cell biology, 2(4), 285-293.
- Turner, R. M. (2005). Moving to the beat: a review of mammalian sperm motility regulation. Reproduction, Fertility and Development, 18(2), 25-38.
- Uzhachenko, R., Boyd, K., Olivares-Villagomez, D., Zhu, Y., Goodwin, J. S., Rana, T., & Ivanova, A. V. (2017). Mitochondrial protein Fus1/Tusc2 in premature aging and age-related pathologies: critical roles of calcium and energy homeostasis. Aging (Albany NY), 9(3), 627.
- Visconti, P. E., & Kopf, G. S. (1998). Regulation of protein phosphorylation during sperm capacitation. Biology of reproduction, 59(1), 1-6.
- Walensky, L. D., & Snyder, S. H. (1995). Inositol 1, 4, 5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. The Journal of cell biology, 130(4), 857-869.
- Wang, C., Wang, Z., Xiong, Z., Dai, H., Zou, Z., Jia, C., & Chen, Z. (2016). mTORC1 activation promotes spermatogonial differentiation and causes subfertility in mice. Biology of reproduction, 95(5).
- Wang, L., Harris, T. E., & Lawrence, J. C. (2008). Regulation of proline-rich Akt substrate of 40 kDa (PRAS40) function by mammalian target of rapamycin complex 1 (mTORC1)-mediated phosphorylation. Journal of Biological Chemistry, 283(23), 15619-15627.
- WHO: World Health Organization (2010). Laboratory manual for the examination and processing of human semen fifth edition.
- Wiza, C., Nascimento, E. B., & Ouwens, D. M. (2012). Role of PRAS40 in Akt and mTOR signaling in health and disease. American Journal of Physiology-Endocrinology and Metabolism, 302(12), E1453-E1460.
- Xie, F., Garcia, M. A., Carlson, A. E., Schuh, S. M., Babcock, D. F., Jaiswal, B. S., & Conti, M. (2006). Soluble adenylyl cyclase (sAC) is indispensable for sperm function and fertilization. Developmental biology, 296(2), 353-362.
- Xiong, M., Ferder, I. C., Ohguchi, Y., & Wang, N. (2015). Quantitative analysis of male germline stem cell differentiation reveals a role for the p53-mTORC1 pathway in spermatogonial maintenance. Cell Cycle, 14(18), 2905-2913.
- Xu, H., Shen, L., Chen, X., Ding, Y., He, J., Zhu, J., & Liu, X. (2016). mTOR/P70S6K promotes spermatogonia proliferation and spermatogenesis in Sprague Dawley rats. Reproductive biomedicine online, 32(2), 207-217.

- Yoshinaga, K., & Toshimori, K. (2003). Organization and modifications of sperm acrosomal molecules during spermatogenesis and epididymal maturation. Microscopy research and technique, 61(1), 39-45.
- Zalata, A. A., Ahmed, A. H., Allamaneni, S. S., Comhaire, F. H., & Agarwal, A. (2004). Relationship between acrosin activity of human spermatozoa and oxidative stress. Asian J Androl, 6(4), 313-318.
- Zaneveld, L. J. D., & De Jonge, C. J. (2013). Mammalian Sperm Acrosomal Enzymes. A Comparative Overview of Mammalian Fertilization, 63.
- Zegers-Hochschild, F., Adamson, G. D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., & Van der Poel, S. (2009). The international committee for monitoring assisted reproductive technology (ICMART) and the world health organization (WHO) revised glossary on ART terminology. Human reproduction, 24(11), 2683-2687.
- Zegers-Hochschild, F., Nygren, K. G., Adamson, G. D., de Mouzon, J., Lancaster, P., Mansour, R., & Sullivan, E. (2006). The ICMART glossary on ART terminology. Human Reproduction, 21(8), 1968-1970.
- Zini, A., Buckspan, M., Jamal, M., & Jarvi, K. (1999). Effect of varicocelectomy on the abnormal retention of residual cytoplasm by human spermatozoa. Human Reproduction, 14(7), 1791-1793.
- Zini, A., Defreitas, G., Freeman, M., Hechter, S., & Jarvi, K. (2000). Varicocele is associated with abnormal retention of cytoplasmic droplets by human spermatozoa. Fertility and sterility, 74(3), 461-464.
- Zhong-Chong, Z. (2015). mTOR: a novel therapeutic target for diseases of multiple systems. *Current drug targets*, *16*(10), 1107-1132.
- Zumoffen, C. M., Caille, A. M., Munuce, M. J., Cabada, M. O., & Ghersevich, S. A. (2010). Proteins from human oviductal tissue-conditioned medium modulate sperm capacitation. Human reproduction, 25(6), 1504-1512.

6. Appendix

6.1. Appendix 1

Informed Consent

CONSENTIMENTO INFORMADO

Eu, _____

(nome), nascido a _____/_____/_____, com o número de Identificação Civil _____, declaro que autorizo que a amostra biológica seja utilizada para espermograma e uma análise complementar de diagnóstico (estudo de marcadores moleculares) de forma gratuita que em nada prejudica o decurso natural do exame. Os dados sujeitos a sigilo médico só poderão ser revelados a familiares e respetivos médicos com a minha permissão e nunca de terceiros. Poderei revogar este consentimento num prazo de 3 meses sem qualquer explicação. Declaro ainda que os resultados dos testes/dados clínicos poderão/não poderão (riscar o que não interessa) ser usados em publicações científicas de forma anónima e que a amostra poderá/não poderá (riscar o que não interessa) ser utilizada para fins de investigação.

Assinatura

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_____/_____/_____

6.2. **Appendix 2:** Basic semen parameters distributions by age (Scatter plots).

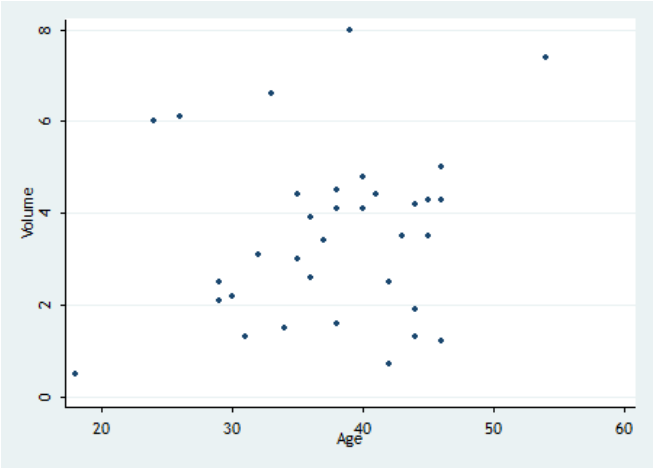

Fig. 1 - Spermatozoa volume distribution by age.

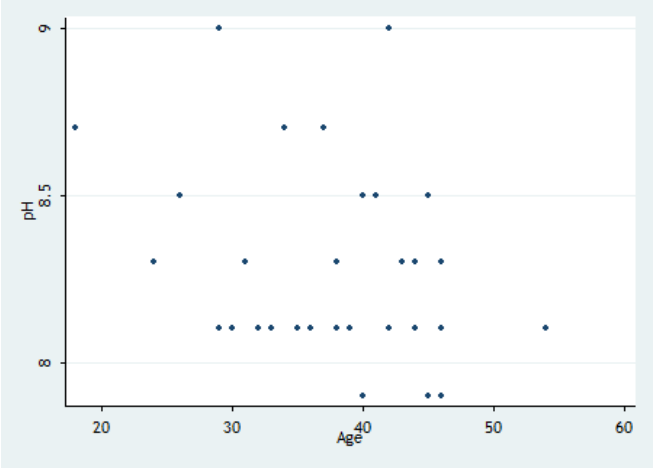

Fig. 2 - Spermatozoa pH distribution by age.

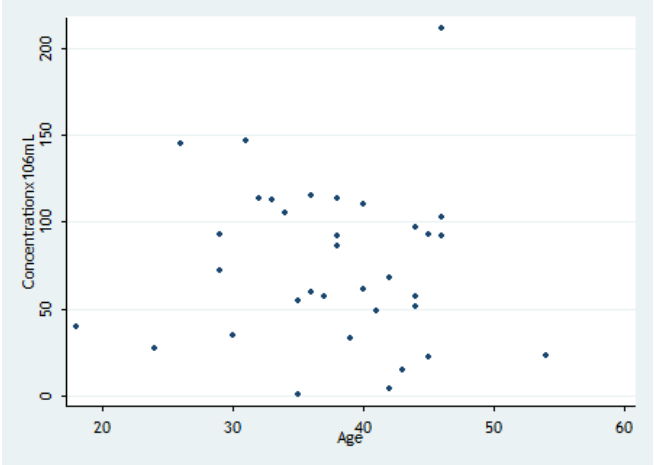

Fig. 3 - Spermatozoa concentration distribution by age.

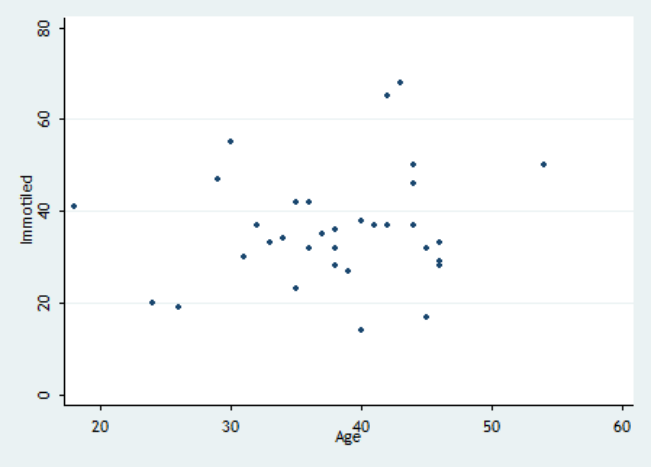

Fig. 4 - Immotile spermatozoa (*type d*) distribution by age.

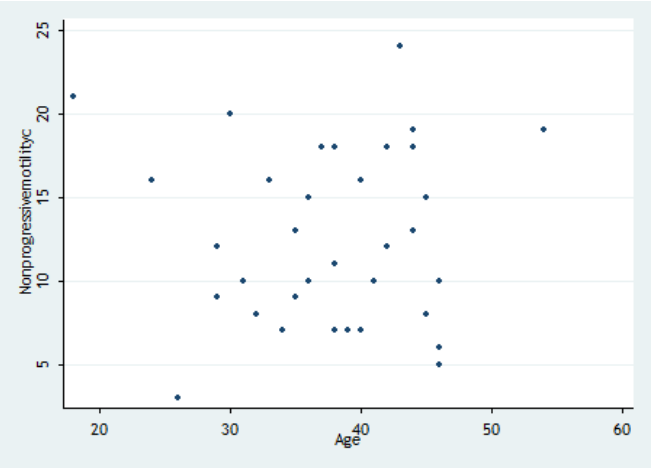

Fig. 5 - Non-progressive motility spermatozoa (*type c*) distribution by age.

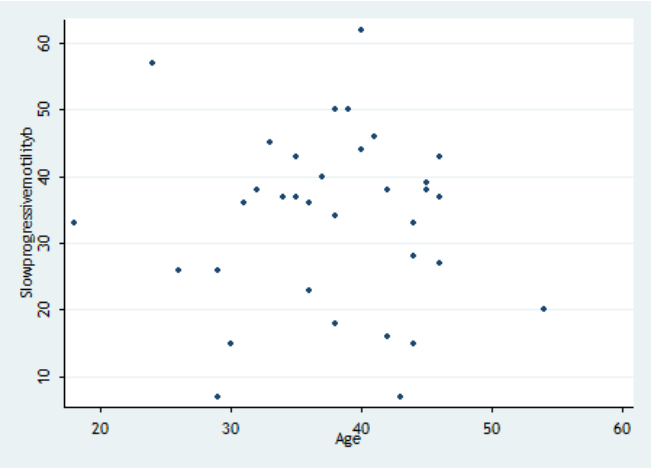

Fig. 6 - Slow progression motility (*type b*) spermatozoa distribution by age.

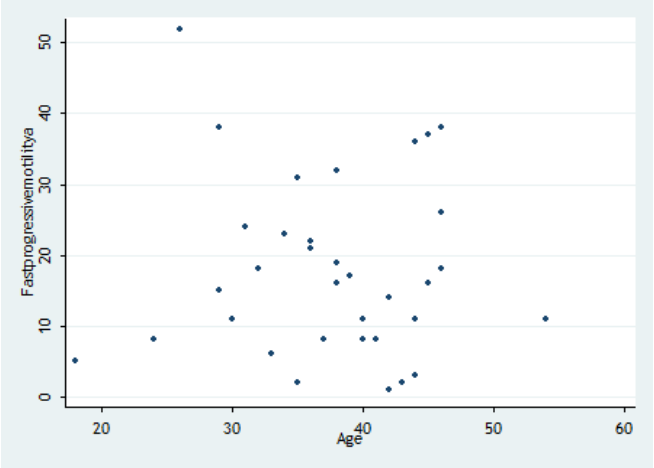

Fig. 7 - Fast progressive motility spermatozoa (*type a*) distribution by age.

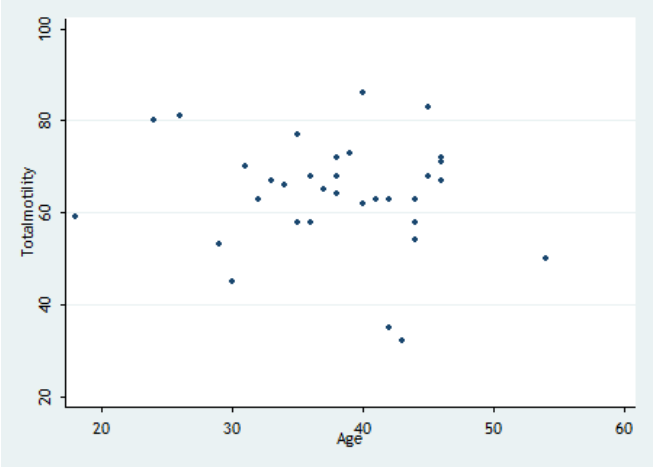

Fig. 8 - Total motility spermatozoa distribution by age.

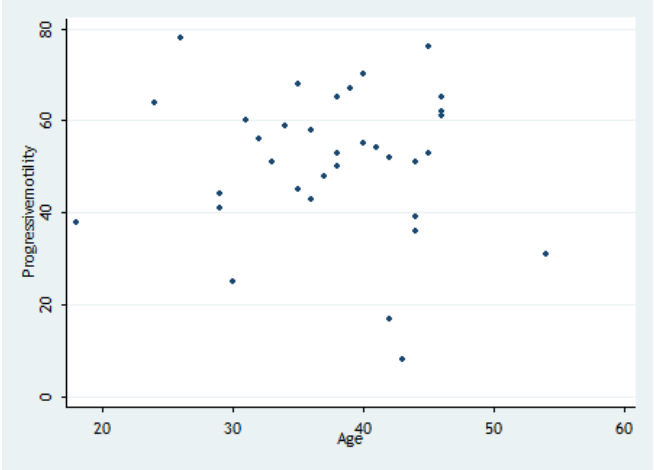

Fig. 9 - Progressive motility spermatozoa (*type a and b*) distribution by age.

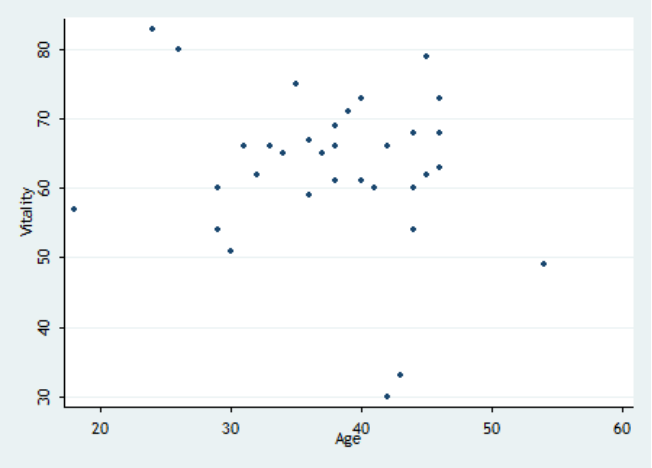

Fig. 10 - Spermatozoa vitality distribution by age.

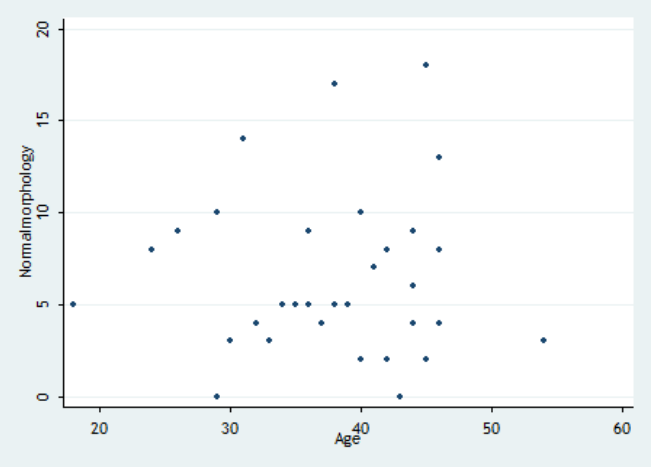

Fig. 11 - Spermatozoa normal morphology by age.

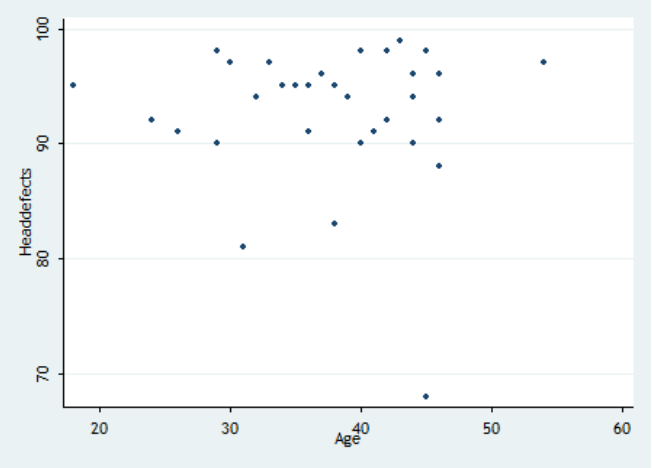

Fig. 12 - Head defects spermatozoa distribution by age.

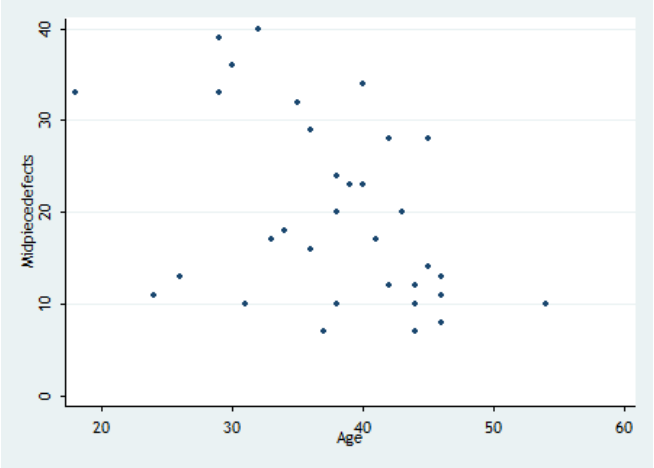

Fig. 13 - Spermatozoa midpiece defects by age.

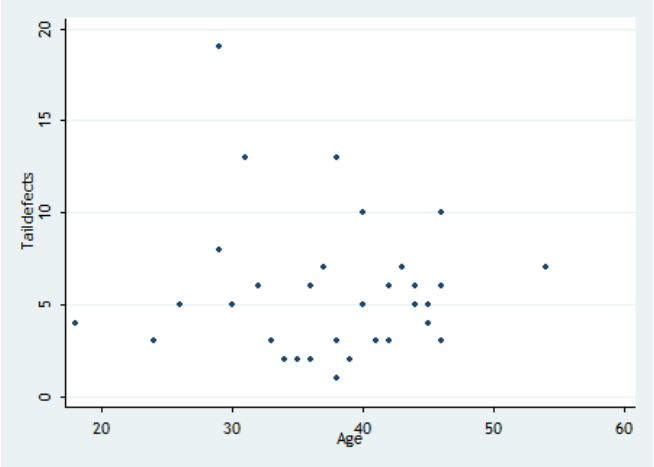

Fig. 14 - Spermatozoa tail defects distribution by age.

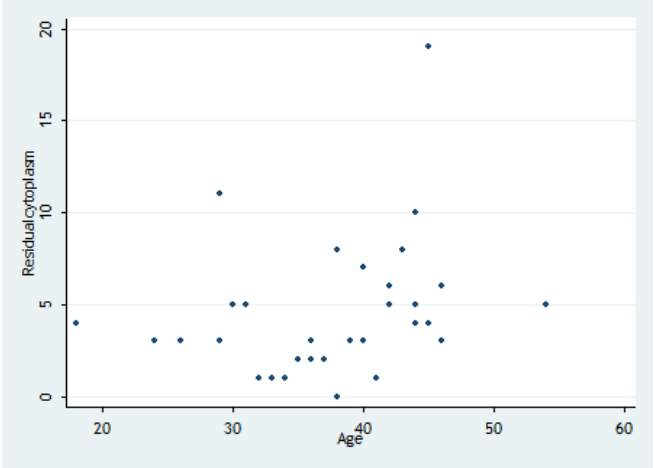

Fig. 15 - Residual cytoplasm spermatozoa distribution by age.

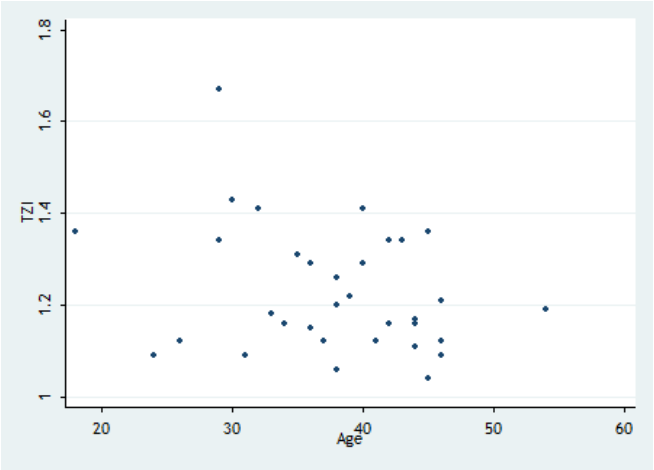

Fig. 16 - Leukocytes concentration distribution by age.

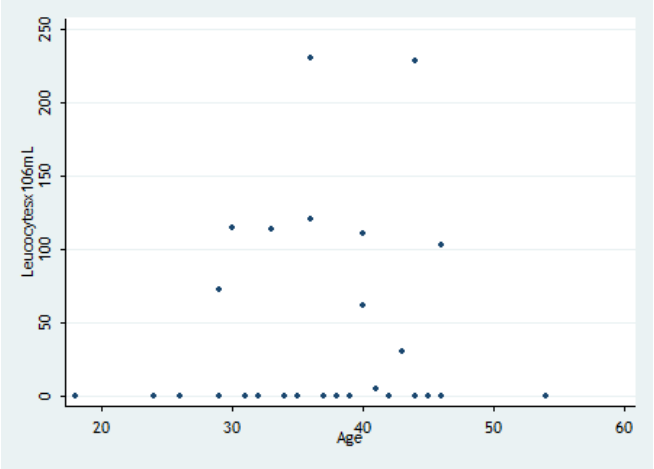

Fig. 17 - Teratozoospermia index (TZI) distribution by age.

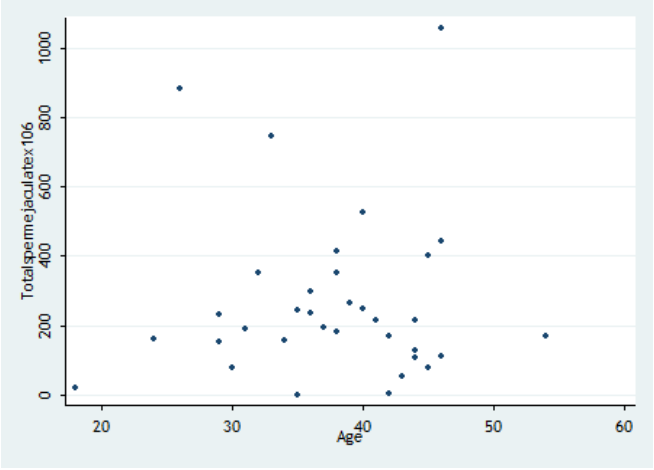

Fig. 18 - Total spermatozoa ejaculate distribution by age.